CCLXVII. BLOOD SUGAR AND BLOOD CHLORIDE CHANGES IN THYROIDECTOMIZED RATS FOLLOWING EXPOSURE TO VARIOUS DAMAGING AGENTS

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Previously described experiments have shown that exposure of animals to non-specific damaging agents, such as toxic doses of drugs, surgical shock, excessive muscular exercise, cold etc., elicits a syndrome with characteristic changes in various organs and in blood chemistry. These changes are largely independent of the specific nature of the damaging agent to which the organism is exposed. However, if an animal is subjected beforehand to a damaging stimulus for a few days, it will no longer respond in this manner to further treatment with the same agent. It was concluded, therefore, that the syndrome represents the somatic expression of the "alarm" of the organism when first confronted with a stimulus to which it is quantitatively or qualitatively not adapted. For this reason, the syndrome has been termed the "alarm reaction" [Selye, 1937, 1, 2; 1938, 1; Harlow & Selye, 1937].

Among the blood chemical changes which characterize the alarm reaction, an initial increase followed by a marked decrease in blood sugar was found to be very constant. With continued treatment this hypoglycaemia disappears within a few days and after resistance to the stimulus is acquired, further treatment will actually increase the blood sugar above the normal level. The blood chlorides show a marked decrease during the alarm reaction and this is followed by an increase after adaptation [Selye, 1938, 2, 3].

It was observed furthermore that removal of endocrine glands which influence the adaptability of the organism may considerably alter this chemical response in the blood. Thus adrenalectomized [Selye, 1938, 4] or hypophysectomized [Selye & Foglia, 1938] animals show no initial hyperglycaemia but a very pronounced hypoglycaemia when exposed to agents capable of eliciting an alarm reaction. The hypoglycaemia is usually most marked in animals which are particularly severely damaged, hence it was not surprising to note an especially marked decrease in the blood sugar in hypophysectomized or adrenalectomized animals, since animals deprived of these glands become unusually sensitive to any damaging agent. It was very unexpected, however, to find in the course of these experiments that removal of the thyroid has an exactly opposite effect, inasmuch as it exaggerates the hyperglycaemic phase of the response. It was noted furthermore that in a group of thyroidectomized rats, the most severely damaged individuals are usually the ones which show the most pronounced hyperglycaemia. In spite of this inverse blood sugar response, the blood chlorides react in the usual manner; in fact, the hypochloraemia is exceptionally pronounced. The purpose of this communication is to report on a series of experiments illustrating these points.

Methods

The blood sugar determinations were carried out by the Shaffer-Hartmann-Somogyi method and the blood chloride determinations by the Van Slyke method. The chlorides were determined in whole blood specimens, not in plasma, because in some cases it was found that a severe alarm reaction may be accompanied by intravital haemolysis. In every case the animals were fasted 24 hr. prior to the determination so as to avoid any change in blood composition resulting from the ingested food. One animal was used for one determination only, so as to avoid changes resulting from repeated fasting and bleeding.

Female "hooded" rats weighing 95–135 g. were used in all the experiments of this series. The alarm reaction was elicited by exposure to cold, formaldehyde treatment or muscular exercise. The animals treated with formaldehyde received 0·2 ml. of a 4% solution subcutaneously three times the first day and once the next morning, 23 hr. after the first injection. They were killed one hour after the last injection. Those exposed to cold were placed in an ice-box at +3° for a period of 24 hr. Those forced to perform muscular exercise were placed in drum cages having a diameter of 12 in. and revolving at a speed of 18 r.p.m. They were forced to make three 15 min. runs in the course of the first day, and one the following morning. They were killed 1 hr. after the beginning of this last exercise period. The total length of treatment was 24 hr. in each case, during which period no food was allowed. This length of treatment was chosen because in normal animals the blood sugar curve usually reaches a low point at about 24 hr. after exposure to an alarm reaction-eliciting agent. 8 normal and 8 thyroidectomized rats were exposed to each of the agents and two similar groups of otherwise untreated rats served as controls. The thyroidectomies were performed 9 days before the beginning of treatment. It should be emphasized that in the rat the two internal parathyroids are enclosed in the thyroid tissue and were consequently removed together with the thyroid. Although no signs of tetany were evident in animals thus operated upon, it is possible that ablation of these parathyroids may have influenced the response. Experiments are now in progress in this laboratory in which only the parathyroids are removed. These may throw some light on this aspect of the problem.

Results

Table I summarizes the results of these experiments. The numbers in parentheses represent extreme variations.

Table I

	Blood sugar, mg. per 100 ml.		Blood chlorides, mg. per 100 ml.	
Damaging agent	Normal	Thyroidectomized	Normal	Thyroidectomized
Formaldehyde	**76** (70–85)	**111** (81–144)	**386** (363–421)	**356** (339–374)
Exercise	**70** (56–80)	**78** (65–82)	**463** (456–479)	**407** (385–410)
Cold	**74** (55–80)	**91** (70–112)	**407** (386–433)	**384** (362–395)
Controls	**85** (81–92)	**70** (60–80)	**435** (421–456)	**423** (410–433)

It is quite obvious that exposure to the various damaging agents used in this experimental series led to a marked fall in the blood sugar of the normal animals, while in the thyroidectomized rats exposure to these same stimuli not only failed to produce such an effect but actually caused hyperglycaemia. This is particularly interesting in the case of animals exposed to cold or performing muscular exercise, since these stimuli call for increased combustion of carbohydrate

reserves. It should also be emphasized that following a 24 hr. fasting period, the blood sugar of thyroidectomized rats is 15 mg. per 100 ml. below that of the normals, a fact which we have confirmed in the course of many experiments performed in connexion with other problems. In spite of this lower initial value, the blood sugar of the damaged thyroidectomized rat is far above that of the normal. The blood sugar concentration was usually highest in those rats which appeared to be most severely damaged. The blood chlorides of the thyroidectomized rats, on the other hand, decrease even more markedly than those of the normals. A particularly interesting point in this connexion is the behaviour of the blood chlorides in the exercised group. Previous experiments have shown that whilst an alarm reaction produced by any damaging stimulus so far examined causes hypochloraemia, that elicited by excessive muscular exercise results in a pronounced rise in blood chlorides [Selye, 1938, 3]. It was found, however, that the same amount of exercise which increases the blood chlorides in the normal rat causes hypochloraemia in the adrenalectomized animal [Selye, 1938, 4]. A similar change in the response was also observed in the thyroidectomized rats of the present series, as will be seen from the data in Table I; the blood chlorides of the normals rose while those of the thyroidectomized rats fell under the influence of the same amount of muscular exercise.

Since prolonged fasting also elicits typical signs of the alarm reaction (hyperplasia of the adrenal cortex, loss of adrenaline from the medulla, atrophy of the thymus, lymph glands and spleen etc.), it seemed of interest to examine the effect of this stimulus on the blood sugar of thyroidectomized rats. 24 normal and 24 thyroidectomized rats comparable in every respect with those in the above-mentioned experiments were used to elucidate this point, one-third of them having been fasted for 24, another third for 48 and the remainder for 72 hr. The results of the blood sugar determinations in these groups are summarized in Table II and Fig. 1.

Table II

Fasting period hr.	Blood sugar, mg. per 100 ml.	
	Normal	Thyroidectomized
24	**85** (80–91)	**70** (61–79)
48	**76** (69–81)	**94** (83–112)
72	**62** (46–69)	**65** (55–76)

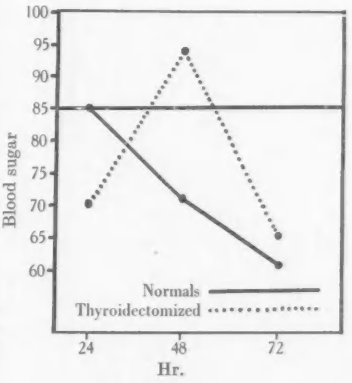

Fig. 1.

It will be seen, in conformity with what we said above, that after 24 hr. fasting, the blood sugar of the thyroidectomized animal is considerably below that of normal controls fasted for the same length of time. Yet after 48 hr. fasting, when morphological signs of the alarm reaction become evident, the blood sugar rises, not only above that of normal controls fasted for the same period, but even above that of normal animals fasted for 24 hr. only. At the end of 72 hr., however, the blood sugar values of both groups are very low and not significantly different from each other. We do not wish to speculate concerning

the fundamental disturbances in the carbohydrate metabolism caused by thyroidectomy on the basis of mere blood sugar determinations, since much more detailed metabolic studies would be required before one could discuss this question to advantage. Yet it seems obvious that the rise in blood sugar is elicited by the non-specific damage inflicted upon the animal and not by the specific action of any of the agents employed; otherwise we should not have obtained the same result with such different agents as formaldehyde, cold, muscular exercise and even fasting.

It is hardly within the scope of this communication to review the existing rather voluminous and contradictory literature on blood sugar changes following thyroidectomy. It is evident however that since a short period of fasting decreases, while a longer period of fasting or exposure to any other damaging agent increases, the blood sugar of the thyroidectomized animal, all experiments designed to study the specific effect of a certain agent on the blood sugar of a thyroidectomized animal necessarily had to be complicated by this particular change in the response to the non-specific harmful stimulus.

Summary

Thyroidectomized rats respond with hyperglycaemia to non-specific damaging agents which decrease the blood sugar of the normal rat.

While a 24 hr. fasting period causes more pronounced hypoglycaemia in thyroidectomized than in normal rats, 48 hr. of fasting actually increase the blood sugar of the thyroidectomized rat while in the normal it causes a further decline in blood sugar concentration.

Thyroidectomized rats exposed to non-specific damaging agents respond with a more pronounced decrease in blood chloride than normal animals. They show hypochloraemia even under the influence of excessive muscular exercise which raises the blood chloride of the normal rat.

The author is greatly indebted to Messrs K. Nielsen, H. Torunski and C. Rasmussen for their excellent and untiring technical assistance which was of great value in completing these studies.

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CCLXVIII. THE CONCENTRATION OF COENZYME-LIKE SUBSTANCE IN BLOOD FOLLOWING THE ADMINISTRATION OF NICOTINIC ACID TO NORMAL INDIVIDUALS AND PELLAGRINS

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THE recent demonstration that nicotinic acid, or its amide, is the vitamin whose deficiency is responsible for canine black tongue and pellagra is of great theoretical interest, for the pyridine ring plays a role of the first importance in cellular respiration. This was demonstrated by Warburg *et al.* [1935] who found their coenzyme (triphosphopyridinenucleotide designated Co II) to comprise one mol. of nicotinic acid amide, three of phosphoric acid, two of (probably) pentose and one of adenine. The nucleotide in the presence of a suitable protein is reduced by the substrate, then reoxidized indirectly by molecular oxygen; this cycle of oxidation occurs in the pyridine ring. Cozymase (diphosphopyridinenucleotide, Co I) differs from Co II only in possessing one mol. less of phosphoric acid, according to Warburg & Christian [1936] and Euler *et al.* [1936]. The mechanism of its cycle of oxidation is the same.

It is almost certain that all active tissues in the human body contain Co I and Co II, and consequently one would expect that variation in amount of dietary nicotinic acid should lead directly to variation in amount of coenzyme. It is the object of this paper to demonstrate that the concentration of coenzyme, or of some closely related substance in the blood, is sensitive to the ingestion of nicotinic acid, both in normal individuals and pellagrins.

The vitamin action of nicotinic acid was first reported for bacteria. Lwoff & Lwoff [1937] proved that Co I or Co II could replace the *V*-factor of the haemophilic bacteria. At about the same time nicotinic acid was shown to be a growth factor for *Staphylococcus* by Knight [1937] for the diphtheria bacillus by Mueller [1937], and recently for the dysentery bacillus by Koser *et al.* [1938].

Frost & Elvehjem [1937] reported that adenylic acid plus nicotinic acid (1 mg. per day of each) completed a basal diet on which rats did not otherwise grow, and Euler *et al.* [1938] obtained a striking response by the addition of 1 mg. per day of Co I (cozymase). However, rat dermatitis (and chick dermatitis) is not due to a nicotinic acid deficiency according to Dann [1937].

Elvehjem *et al.* [1937; 1938] showed that nicotinic acid or its amide is the vitamin whose deficiency is responsible for black tongue in dogs; this has been confirmed by Street & Cowgill [1937] and others, and extended to a pellagra-like condition in swine by Chick *et al.* [1938]. Pellagra, like black tongue, has yielded to nicotinic acid therapy according to Fouts *et al.* [1937], Smith *et al.* [1937], and Spies *et al.* [1938]. Schmidt & Sydenstricker [1938] reported relapses following rapid improvement with nicotinic acid therapy, but neither the diet nor the activity of the patients was controlled, so that at present their results are inconclusive [*vide* also review by Sebrell, 1938].

Method

The concentration of coenzyme-like substance, which is confined to the corpuscles, was determined with the aid of *Haemophilus parainfluenzae*, the growth of which under proper experimental conditions is proportional to the coenzyme content of the culture broth.

Specificity of test

Lwoff & Lwoff [1937] suggested the use of *H. parainfluenzae* for the bio-assay of coenzyme when they found that:

(1) Either Co I or Co II at concentrations as low as about 2×10^{-9} *M* can replace the *V*-factor required for growth by all *parainfluenzae*.

(2) *V*-factor cannot be replaced by adenylic acid (yeast or muscle), nicotinic acid, nicotinamide, diethylamide, *o*-dihydropropylnicotinamide, or the products formed when either coenzyme is kept at 100° and *p*H 8·5 for 20 min.

(3) The reaction Co I $\rightleftharpoons$ Co II can be accomplished by the bacteria when either coenzyme enters the protoplasm, after which its identity appears to remain fixed.

I have found that neither nicotinic acid nor its amide in therapeutically active concentrations, either alone or in the presence of all substances found in the blood extract used for assay, is able to act as *V*-factor, as the following examples show. (A) The addition to blood extract of 1 mg. acid or amide per ml. blood reduced the assay by 4 and 6% respectively. (B) Incubation of whole blood containing 1 mg. acid per ml. at 37·8° for 1 hr. increased the assay by 6%. (C) Incubation of whole blood containing 10 mg. acid per ml. rapidly produced methaemoglobin, haemolysis and loss of *V*-factor.

Products of the mild acid hydrolysis of *V*-factor were found to be inactive, as when blood, diluted 100 times, was boiled for 20 min. in 0·8 *N* trichloroacetic acid containing 0·55 *N* NaOH.

These results are in harmony with those of the Lwoffs; they argue strongly in favour of the unique identity of Co I and Co II with *V*-factor, and prove that only a special and limited group of compounds containing nicotinic acid, or its amide, can be active. However, the terms coenzyme and *V*-factor will not be used interchangeably in the following, and the substance or substances measured in the bio-assay will be referred to as *V*-factor.

Theory of test

When broth is inoculated, the optical density of the resulting suspension increases owing to an increase in living and dead bacterial matter, and to changes in the medium itself. The increase in light adsorption is rapid at first, but reaches a maximum at which it tends to remain 24–30 hr. after inoculation, declining slowly thereafter. To measure directly the change in light transmission of a culture it is most convenient to employ the Evelyn photoelectric colorimeter [Evelyn, 1936], which uses test tubes for absorption cells.

Let T_b be the transmission of the blank culture, and
T_x the transmission of the experimental; then
a the % change in absorption is given by (1):

$$a = 100 \left[1 - \frac{T_x}{T_b}\right]. \qquad \text{.....(1)}$$

V-content is found by determining the amount of blood necessary to produce a value of a equal to that produced by a standard solution.

Let a_s be some set value of a used as a point of reference,

X the concentration (ml./ml.) of blood in the unknown test whose reading is a_s,

S the concentration (ml./ml.) of standard V-factor in the standard test whose reading is a_s, and

H the haematocrit reading (volume of red and white cells expressed *as* $^0/_0$ *blood volume*); then

V the concentration (units/ml.) of V-factor in the blood cells, expressed in arbitrary units and multiplied by 10^4 for convenience, is given by (2):

$$V = \frac{10^4 S}{HX}. \qquad \text{.....(2)}$$

V determined according to (2) will be used as an empirical measure of the concentration of Co I and Co II, bearing in mind, however, that some unknown substance closely related to the coenzymes might give a positive test. No attempt was made to distinguish between the two coenzymes.

Procedure of test

The cultures are prepared in tubes suitable for use in the photoelectric colorimeter, each containing 7 ml. broth. To one series of tubes in duplicate there are added 0·0, 0·1, 0·1, 0·2 and 0·3 ml. blood extract; to a second series there are added similar amounts of standard V-factor solution. All tubes are inoculated except the second pair in each series. The tubes are checked in the photometer (using filter 520 M) for initial differences in absorption, which are noted and used as corrections. After 24 and 30 hr. of incubation at 37° they are read in the photometer; the maximum value of a is used, and the averages for each pair are plotted as in Fig. 1. Usually the difference between the 24 and 29 hr. readings is small and the duplicates agree fairly well as in the following readings made at 24 hr.:

Standard V. 0·1 ml., 2, 1·5; 0·2 ml., 7, 7; 0·3 ml., 11·7, 11.

Blood extract. 0·1 ml., 3·3, 4; 0·2 ml., 11·2, 12·2; 0·3 ml., 14, 13.

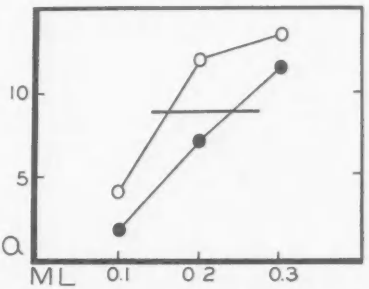

Fig. 1. a as a function of V-factor (in ml.). Open circles, V-factor from blood extract; solid circles, V-factor from yeast extract used as a standard. The bar cutting the two curves at $a=9$ (designated a_s) facilitates comparison: 0·16 ml. blood extract is equivalent to 0·245 ml. yeast standard.

The horizontal bar cutting the two curves in Fig. 1 represents a_s, i.e. the value of a used in comparing the unknown and standard solutions. a_s equal to 9 has been used throughout the present work. In Fig. 1 we find by interpolation that 0·16 ml. blood extract is equivalent to 0·245 ml. standard V-factor solution (for preparation and concentration, see below), each producing a change equal to a_s. From these data X and S are calculated, and knowing H the value of V is obtained from (2).

Blood extract. Blood obtained by venepuncture is prepared by the addition of 1·2 mg. potassium oxalate per ml. 0·1 ml. blood is added to 10 ml. distilled water. Immediately 2 ml. are withdrawn for the haemoglobin determination [Evelyn, 1936] and are replaced by 2 ml. trichloroacetic acid solution (13% acid containing 2·15% NaOH). The test tube is plugged, centrifuged after several minutes and stored on ice. The V-factor is contained in the supernatant liquid, which loses 5% of its activity in 24 hr. Plasma prepared in this manner supports no measurable growth, even when 1 ml. instead of 0·1 ml. is used.

Cultivation of bacteria

The bacteria used were of the same strain as those employed by the Lwoffs, namely, *H. parainfluenzae* strain 4101 Fleming, National Collection of Type Cultures, Lister Institute, London. Subcultures were made at least three times a week, alternating between broth and agar. When first received, the bacteria showed 50–100% more growth if glucose or sucrose were added to the medium. After $3\frac{1}{2}$ months of cultivation at 37°, however, this phenomenon disappeared rather suddenly, although the ability to oxidize sugar was retained. All data reported here were obtained after the mutation had occurred; during this period cultivation in broth was almost continuous.

V-factor. This is prepared by suspending 100 g. baker's yeast in 50 ml. water, pouring the suspension into 150 ml. boiling water containing 6·8 g. KH_2PO_4, and maintaining the temperature at 80–85° for 20 min. The suspension is then filtered through a bacteriological paper, and sterilized by passing through a candle or a Seitz filter pad. The concentration of this solution is 5×10^{-1}, as 0·5 g. yeast per ml. water has been used. The strength of the *standard solution* referred to in the text and used in all experiments was 6×10^{-4}.

Medium. A 2% solution of proteose-peptone (Difco) containing 0·6% NaCl is titrated with NaOH to *p*H 7·7–7·8, tubed in 6 ml. samples, and autoclaved. Sterile *V*-factor is then added. The usual subculture receives 0·1 ml., concentration 5×10^{-2}. Subcultures intended to supply inocula for blood tests receive 0·1 ml., $2{\cdot}5 \times 10^{-3}$, all of which will be used up after 24 hr. of growth. Thus when the test culture is inoculated with one drop, no *V*-factor will be carried over. For a solid medium, the above is used plus 3% agar (*V* added when agar has cooled, but before solidification).

Experimental results

Figs. 2, 3 and 4 together with Table I summarize the experimental data. The solid black points are plotted for periods during which nicotinic acid was taken; an arrow indicates when the administration of acid began or ended between two points; *V* the ordinate in each graph was calculated according to equation (2), *H* and *S* being redetermined for every point. The abscissae of all are comparable; i.e. a determination made on the twentieth day of Fig. 2 would have been made simultaneously with that on the twentieth day of Fig. 3. This was done to provide a further check on the method.

All normal subjects took nicotinic acid by mouth after breakfast and dinner either in tablets (Squibbs) or in solution (S.M.A. Corp. nicotinic acid dissolved in water). A 0·5% solution (partially neutralized) has a slightly sour taste and is not unpleasant. When 100 mg. are taken on an empty stomach (e.g. before breakfast) a peripheral flushing occurs, the extent of which varies in different individuals. Usually within 5 min. the face and neck tingle, then flush, followed by the forearms and probably the knuckles and knees. In some cases intravenous injection was used.

Normal subjects

(1) HK (Fig. 2), white male, age 28, height 179 cm., weight 66·5 kg. During first 10 days, average *V* was 19. From 10th to 24th day about 100 mg. nicotinic acid were taken daily, the change in *V* being as follows: 16th day, total nicotinic acid 475 mg., +20%; 19th day, 825 mg., +40%; 22nd day, 1500 mg., using smooth curve +50%.

Beginning with the 24th day nicotinic acid was discontinued, and the subject was placed on a pellagra-producing diet (defined below). The increase in *V* declined: 32nd day, +10%; 40th day, +0%. Beginning on the 43rd day 1200 mg. nicotinic acid were taken within 24 hr. (300 mg. at 11.30 a.m., 2 p.m., 6.15 p.m. and 10.30 a.m.): 8 hr. after first dose *V* rose +10%; 24 hr. (44th day), +40%; 48 hr. (45th day), +50%; 5 days (48th day), +30%; 9 days (52nd day), +5%.

The pellagra diet used was similar to that of Ruffin & Smith [1937], but with the following changes: *omitted*, cod liver oil, iron ammonium citrate, calcium gluconate, field (cow) peas, lard and flour; *decreased*, ascorbic acid to 50 mg. per day; *added* per day, 1 mg. aneurin-Cl; 5 drops oleum percomorphum, 200 g. potatoes, 5 stewed prunes, 70 g. apple sauce, 20 g. butter. The modified diet, as compared with the original, contains less preventive substance [cf. food ratings of Sebrell, 1934], but is low in protein.

(2) RM (Fig. 3, open circles), white male, age 24, weight 70 kg. Average initial V was 17 during the 13th–16th days. During 16th–23rd days about 100 mg. nicotinic acid taken daily. The changes in V together with the total dose were: 20th day, 400 mg., +30%; 23rd day, 1 g., smooth curve +35%; thereafter nicotinic acid stopped, 34th day, −5%. Beginning on the 50th day 1·25 g. nicotinic acid were taken during 24 hr. The changes in V were: 50th day (after 250 mg.), +10%; 52nd day, +30%; 55th day, +25%.

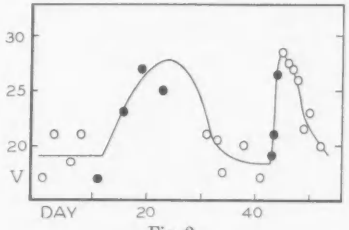

Fig. 2.

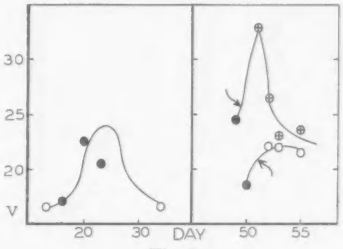

Fig. 3.

Fig. 2. Variation of V in the normal subject HK. 1·5 g. nicotinic acid taken during the 10th–24th days produced an increase of 50%. 1·2 g. taken within 24 hr. on the 43rd day produced a similar effect. Solid circles indicate periods during which nicotinic acid was taken.

Fig. 3. Variation of V in the normal subject RM (open circles) and the pellagrin JH (crossed circles). Solid circles indicate the administration of nicotinic acid, which was stopped at the arrows. RM (left curve) received 1 g. nicotinic acid in 7 days during which V rose 35%. Later (right-hand curve) RM received 1·25 g. in 24 hr., and V rose 30%. JH the pellagrin received 700 mg. nicotinic acid within 48 hr. ending at the arrow. Note the rapid rise in V of 35%, and the rapid fall.

The remaining four white male subjects, whose data are graphed together in Fig. 4C, will be considered as a group. Ages, weights and graph symbols are as follows: FE, 23, 66, circle; GS, 24, 61·5, triangle; KB, 22, 79·5, square; WK, 22, 97·5, inverted triangle. The daily dose of acid was about 1·5 mg. per kg.; FE and GS took 100 mg. per day, KB 125 and WK 150.

The data show an average initial V of 18 during the 13th–16th days. About 1·5 mg. nicotinic acid per kg. were taken daily during the 16th–20th days, and twice this dose for the 21st and 22nd days. The increases in V were: 20th day, 40%; 23rd day, 45%; 35th day, 10%.

Pellagrins

(1) JH, white male, age 55, weight 40 kg. The patient had suffered from oesophageal obstruction for 25 years. For 6 months before admission almost all food eaten had been regurgitated within 5–20 min.; as a result he was quite emaciated. About 2 weeks before admission definite signs of pellagra had appeared. On admission, physical examination showed the dorsum of each hand to be red and rough, and the tibial surfaces of the legs to be discoloured with a brownish pigmentation. The lips were dry, the tongue very red and smooth, the buccal surfaces red, the pharynx red. Oesophageal dilatation was performed with marked relief of the obstruction, and the patient was put on a high caloric, high vitamin diet. Valentine's liver extract, 30 ml., t.i.d., and nicotinic acid, 20 mg., t.i.d., were given for 4 days, then stopped. On the 5th and 6th days 300 and 400 mg. nicotinic acid were given. Eight days later acid and liver extracts were restored to the diet.

Data for JH are plotted in Fig. 3, crossed circles; the arrow marks the end of 2 days during which 700 mg. nicotinic acid were taken. At the time of the first test 600 mg. had been taken already, and V was 24·5 (49th day on graph). Note the subsequent sharp rise and fall: +35%, 51st day; +8%, 52nd day. The true initial value of V is not known; hence the increase (see Table I) is a minimum. The patient's course was one of steady improvement. The signs of dermatitis disappeared almost entirely within 3 weeks.

The following cases were kindly brought to my attention by Drs D. T. Smith and J. M. Ruffin; they constitute part of a series studied in other respects by these workers, which will be described

elsewhere. The basal diet referred to [Ruffin & Smith, 1937] contained no vitamin fortification and a minimum of *pp* factor.

(2) RC, white male, age 41, weight 61·9 kg., height 176 cm. Three weeks before admission, after exposure to sun, dermatitis developed on his hands, and was followed 1 week later by stomatitis and glossitis. Diet was fair, but lacking in meat. There was no history of alcoholism. On admission, physical examination showed the skin of the dorsa of the hands to be reddened, cracked and peeling. "Sand paper skin" was noted on both sides of the nose around the sebaceous glands. The gums were sore and tender, the mucous membranes, pharynx and tongue were red. Gastric analysis revealed no free HCl at any time. Gastroscopy showed a reddened mucosa without ulcers or erosions.

RC was placed on the basal diet and given graduated doses of sunlight to the right hand. After 3 days his tongue became red and swollen, the dorsum of the hands inflamed, and a mild diarrhoea developed. During this period the average *V* (Fig. 4B, circles) was 15. Nicotinic acid administration then began (3rd day in Fig. 4B), 90 mg. per day i.v. After the second dose an improvement could be noted in the glossitis, and from then on the patient improved steadily, although the sebaceous glands over the nose were tardy in responding. According to Fig. 4B the increases in *V* correlated to total dose of nicotinic acid were: 6th day, 270 mg., 40%; 9th day, 540 mg., 75%; 12th day, 810 mg., 85%.

In Fig. 4A there are compared the results obtained with two subjects on the same hospital diet and with same initial value of *V*, one of whom received nicotinic acid.

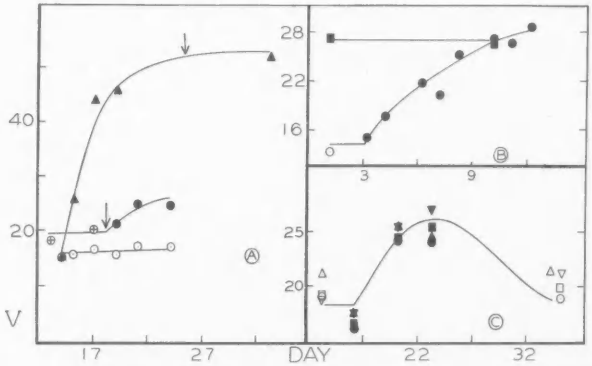

Fig. 4. Variation in *V* following the administration of nicotinic acid (indicated by solid circles and arrows). Fig. 4A, triangles, pellagrin JS, 1 g. daily, *V* rose 250%; crossed circles, pellagrin LL, 60 mg. daily, *V* rose 30%; open circles, pellagrin RJ, spontaneous recovery, *V* rose 5%. Fig. 4B, circles, pellagrin RC, 90 mg. daily, *V* rose 100%; squares, pellagrin JA, received 5·6 g. during 26 days before first determination, and a total of 8·8 g. at time of the second. Fig. 4C, normal subjects, circle, FE; triangle, GS; square, KB; inverted triangle, WK. Each took 1–1·5 g. nicotinic acid. The average rise in *V* was 45%.

(3) RJ, white male, age 55, weight 61·3 kg., height 169 cm. The diagnosis was pellagra, hypertensive cardiovascular disease and arteriosclerosis. Inquiry revealed that he had had an apoplectic seizure 17 months previously, following which his physician had ordered the elimination of meat from the diet. There was also a lack of fruit. Two weeks previously after exposure to the sun for several hours the signs of pellagra had appeared. When admitted, the dorsal skin of each hand was fiery red and desquamating, with oedema of the subcutaneous tissue. The bowels were costive. Glossitis was not present.

The data for RJ are plotted in Fig. 4A, open circles. When admitted, he was placed on the basal diet and exposed to the sun for several days without effect. The dermatitis improved steadily, and he was finally discharged some 20 days later. The initial value of *V* was 15, and it remained at this level, advancing to 16, an increase of about 5%. The spontaneous recovery may have resulted in part from the eating of a meal of liver following the outbreak of the dermatitis.

(4) JS, white male, age 55, weight 59·9 kg., height 158 cm. The diagnosis was pellagra, hypertensive vascular disease, generalized arteriosclerosis and peripheral neuritis. About 2 years previous to admission, the patient had been treated at this hospital for pellagra with Valentine's liver extract, with excellent results. Upon returning home, however, medication was stopped and meat was eliminated from the diet because of the high blood pressure. One month before admission, he had strolled through the fields in the sun; shortly afterwards dermatitis, stomatitis and diarrhoea developed. Upon admission, dermatitis of the dorsum of the hands was noted, the lips were swollen and sore, the mucous membranes red and dry, the tongue bright red and very slick and the pharynx reddened. There was no diarrhoea, but the patient complained of gastrointestinal symptoms, which continued throughout his course in the hospital. Gastric analysis showed the presence of free HCl after histamine. The patient was put on the basal diet, and 2 days later the oral administration of nicotinic acid was begun, 250 mg., q.i.d. Beginning 3 days after admission, and continuing for 3 successive days, 100 mg. per day of aneurin-Cl were given.

Data for JS are plotted in Fig. 4 A, triangles. The initial value of *V* was 15; graph abscissa, 14th day. Thereafter the total dose of nicotinic acid and increases in *V* were: 15th day, 1 g., 75%; 18th day, 4 g., 190%. Administration of nicotinic acid was stopped on the 25th day, total dose, 11 g. On the 33rd day, the increase in *V* was 245%; probably the increase was even greater around the 27th day.

(5) JA, white male, age 65, weight 56·6 kg., height 177·5 cm. Diagnosis: pellagra, generalized arteriosclerosis, polyneuritis due to beriberi. At time of admission this patient was critically ill. He was afflicted with a marked psychosis, the lesions on his hands were secondarily infected and the stomatis and glossitis were marked. He was put on the basal diet and given nicotinic acid, 90 mg. i.v. daily for 10 days. At the end of this period there was an amazing improvement in his entire condition. Nicotinic acid was then given orally, 90 mg., q.i.d., and also aneurin-Cl.

The first value of *V* for JA (Fig. 4 B, squares) was obtained 26 days after treatment had been begun, at which time the patient had received 5·6 g. nicotinic acid and 450 mg. aneurin-Cl; *V* was

Table I. *Effect of nicotinic acid administration on* V

Subject	Total acid taken	Period of administration	*V*			Duration of increase
			Initial	Maximum	Increase	
Normals	mg./kg.	days	units	units	%	days
HK*	22·5	10	19	28	50	10
	18	1	19	28·5‡	50	8
JM	14·5	7	17	23·5	35	11
	18	1	(17)	22‡	30	—
Average of FE, KB, GS WK	14·5	7	18	26·5	45	8
Pellagrins						
JH	17·5	2	Less than 24·5	33§	More than 35	2
RC†	4·3	3	14	21	50	—
	8·6	6	—	26	85	—
	13	9	—	28	100	—
JS†	17	1	15	26	75	—
	67·5	4	—	44	190	—
	186	11	—	52‖	254	—
LL	8	6	19	25	30	—
JA	215	26	—	26·5	—	—
	250	35	—	26·5	—	—

* After taking first dose of nicotinic acid the subject was maintained on a pellagra-producing diet.
† On Ruffin & Smith basal diet.
‡ 24 hr. after nicotinic acid was discontinued.
§ 36 hr. after nicotinic acid was discontinued.
‖ 8 days after nicotinic acid was discontinued

about 26·5; 9 days later the value was the same, the total doses being 8·8 g. nicotinic acid and 850 mg. aneurin-Cl.

(6) LL, white female, age 40, weight 45·8 kg., height 160 cm. Diagnosis: malnutrition, pellagra, secondary anaemia, pyorrhea alveolaris, chronic cervicitis. Three months before admission there had been a gradual onset of weakness, anorexia and proneness to fatigue; 3 weeks before admission dermatitis began. When admitted, the skin of the face was somewhat reddened, and on the hands and arms to just over the elbows it was rough, brownish and scaling. Similar lesions were present on the ankles.

LL was put on the basal diet and exposed to sunlight, but showed no sensitivity. The data for *V* are plotted in Fig. 4A, crossed circles. The initial value was about 19; after 6 days on a high caloric, high vitamin diet plus 60 mg. nicotinic acid per day (begun at arrow), *V* had risen 30%. The patient's general condition improved and she was discharged.

Discussion

The results presented, which are plotted in the figures and summarized in Table I, establish that *V* is a direct function of nicotinic acid intake. The normal value is about 18 ± 2, and this was increased by as much as 200% following the ingestion of nicotinic acid. Within the range of dose studied (about 20 mg. per kg.), the increase in *V* is independent of the duration of the period of administration (1–10 days).

It is rather surprising, however, that *V* should fall so rapidly to the original basal level when nicotinic acid is discontinued, particularly since no measurable quantities are detected in the plasma. Apparently some fundamental difference exists between the initial *V*-factor found in the corpuscle and the increment which is rapidly added. The basal level is stable and possibly is determined entirely at the time of haematopoiesis, whereas the increment is mobile and reflects the current state of nutrition.

Whether these levels can be used to indicate pellagrous conditions is not certain. It is suggestive that the duration of the increment in the pellagrin JH was 2 days, whereas in the normals it was about 10. The relation between the basal level and pellagra is certainly complicated. For example, RJ and JS (Fig. 4A) had the same initial *V* although the symptoms of JS were very much the worse; RJ made a spontaneous recovery. The few cases reported taken with several others indicate that pellagra is to be suspected when *V* is not more than 15, although the converse may not be true.

No relation between *V* and haemoglobin was noted.

If we accept nicotinic acid as the vitamin whose deficiency causes pellagra, a number of consequences follow.

(1) In pellagra the coenzyme level of the body will be reduced, leading directly to a reduced capacity for oxidations and reductions. The basal metabolism will not necessarily be affected, however, since a fraction of the total coenzyme should be adequate for it, but the maximum rate of work will be decreased.

(2) The more active the organism, the greater will be its nicotinic acid requirement. This conclusion is attractive because it will explain some of the spontaneous recoveries, and also because it partly accounts for the differential susceptibility of individuals on the same diet.

The coenzyme role of nicotinic acid, however, does not appear capable of explaining all of the facts relating to pellagra. As noted previously, the relationship between blood *V*-level and pellagra is not clear. Furthermore, since these experiments were completed, Daft *et al.* [1938] in a preliminary note have

reported that 50 mg. of Co I administered intravenously failed to have a therapeutic effect on black tongue. Thus it seems probable that nicotinic acid enters the metabolism in ways as yet unknown

Summary

The bio-assay of blood *V*-factor was accomplished by means of *Haemophilus parainfluenzae*, as supplied by Lwoff & Lwoff. *V*-factor represents the coenzyme moiety (di- and tri-phosphopyridine nucleotides), and, possibly, closely related substances which are unknown. Nicotinic acid (or amide) does not give the test.

V-factor is confined to the corpuscles. About 20 mg. nicotinic acid per kg. taken in 1–10 days will increase *V* by 35–75% in 2–10 days in normal individuals and pellagrins.

The rapid rise in *V*-factor is followed by a fairly rapid fall to the original level. Apparently, material responsible for the increment in *V* is to be distinguished from that which constitutes the initial corpuscular content.

It is a pleasure to thank Dr F. Bernheim for criticism and help given during the course of this work.

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CCLXIX. MECHANISM OF SYMBIOTIC NITROGEN FIXATION

IV. SPECIFIC INHIBITION BY HYDROGEN[1]

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Because of the practical importance of the problem in agriculture, research on the fixation of atmospheric nitrogen through association of the root nodule bacteria and leguminous plants has been primarily directed towards answering questions of immediate agronomic significance. As a result, advances concerned with the more fundamental aspects of this biological process have lagged behind practical applications. Part of the lag, however, may be ascribed to lack of suitable techniques for investigation of the biochemical phases of the problem. In symbiotic nitrogen fixation the life processes of two organisms, plant and bacteria, are intimately related so that there is great difficulty in isolating the factors which have special significance for the fixation reaction. This difficulty has been overcome in part by Wilson and collaborators [Wilson, 1936; Wilson & Fred, 1937; Wilson & Umbreit, 1937] through the application of a physico-chemical approach to the study of the properties of the enzyme system responsible for the fixation.

In the initial studies, which were undertaken to determine the influence of the pN_2 in the atmosphere on the fixation reaction, it was observed that the partial pressure of N_2 could be lowered to about 0·10–0·15 atm. without decrease in the quantity of N_2 fixed by nodulated red clover plants provided that the N_2 removed was either unreplaced or replaced with helium or argon. If the N_2 were replaced with H_2, however, the total quantity of N_2 fixed decreased linearly with the pN_2 of the atmosphere throughout the range 0·06 to 0·80 atm. As such a finding suggests that H_2 may be a specific inhibitor for the symbiotic N_2 fixation process, detailed studies of the "hydrogen effect" were undertaken.

Of primary importance is the demonstration that the effect of H_2 on the assimilation of free N_2 is quantitatively different from the effect on the uptake of combined forms, as NH_4^+ and NO_3^-. A previous report [Wilson & Umbreit, 1937] discussed the various types of experiment which might be expected to throw light on this question; in the same report data were summarized from experiments in which the *total quantity* of N_2 fixed was used as a measure of the effect of H_2. In this paper other data will be presented from experiments in which the *rate* of fixation is used as a criterion.

Methods

Twenty red clover plants (*Trifolium pratense*, L.) were grown in a N-poor sand substrate placed in 9-l. pyrex pressure bottles. All plant nutrients except combined N were added to the sand. Plants of one series were inoculated with an effective strain of the specific organism, *Rhizobium trifolii*; plants of a second series were not inoculated but furnished periodically with combined forms of N.

[1] Herman Frasch Foundation in Agricultural Chemistry, Paper No. 149.

Under the conditions of the experiments the limiting factor for development of the plants was the supply of N. Each bottle was closed with a gas-tight rubber stopper fitted with connexions for changing the atmosphere given the plants. Details of the methods have been described previously [Wilson, 1936].

General considerations of the experiments

As was emphasized in an earlier paper [Wilson & Umbreit, 1937], rate experiments with plants grown in a controlled environment present technical difficulties associated with restriction of available space and apparatus. In most experiments there were six treatments (three different partial pressures of H_2 with two series of plants); at each harvest cultures from each treatment were harvested in duplicate. Since in this work not more than about 50 plant cultures could be made in any one experiment, the maximum number of harvests which could be taken was four. The period during which the plants were under different atmospheres rarely exceeded a month; at the end of this time the inoculated plants in air (or plants which had been given combined N) completely filled the plant container. As it was inadvisable to make a harvest more frequently than once a week, this consideration once again imposed a limitation of four harvests.

When the quantity of free or combined N assimilated by red clover during the period under study is plotted against time, the resulting curves are logarithmic in form; when plotted on semi-log paper the points fall along a straight line. The following factors will cause minor deviations from this straight line.

(1) *Sampling errors.* In spite of all efforts to maintain the plants in identical environments other than the composition of the atmosphere furnished them, variations between the N content of replicates reached 10–15%. Statistical analysis of a large number of experiments [Wilson, 1936; Wilson & Umbreit, 1937] indicates that the *mean* of duplicate samples has a standard error of 5 to 8%. In rate experiments if only duplicate samples are taken at each harvest, through chance these may be cultures with N content definitely lower (or higher) than the mean of all the samples of that particular treatment. An inaccurate sample at one harvest will affect succeeding samples in an opposite direction since the remaining samples will be higher (or lower) than the general mean. For this reason it is believed that the best straight line which can be drawn through the experimental points probably represents the true course of development of the plants. In calculations, points on this line are taken rather than the actual values derived from the samples.

(2) *Restriction of growth.* As has been mentioned, at the end of the experiment the plants in certain of the treatments almost fill the container. During the last week of growth it is probable that the development of these plants is restricted to some extent because of competition for space. This restriction will cause the observed value for N uptake to be less than it would have been had the competition not occurred. A partial remedy for this may be to grow fewer plants in the container, but a reduction in the number of plants taken for analysis will increase the sampling error so that little is gained. Moreover, a great deal of the restricted growth obtains because the plants have reached the top of the container and are more easily shaded by the stopper and other attachments on the bottle. Decrease in the number of plants per bottle would not correct this cause of restricted growth.

(3) *Change in environment.* In spite of the fact that the experiments are relatively short-time in nature, during certain seasons there may be encountered great variations in the weather which will affect the rate of development of the plants for short time intervals. Little can be done to avoid the vagaries in the climate other than to perform the experiments, in so far as is convenient, during seasons of fairly stable weather conditions.

If the development of the plants is logarithmic with respect to time, it is advantageous to use the specific constant of N assimilation, g, for comparison of effect of treatment [Burk, 1934]. This constant is defined by

$$g = 2{\cdot}303 \frac{d \log (a+y)}{dt} = 2{\cdot}303 \times \text{slope straight line of semi-log graph}$$

in which a equals N content of plants at start of treatment and y equals increase after time t. The value of this constant represents rate of assimilation of N per unit concentration of N and is therefore independent of the time.

Rate experiments

Two experiments have been made in which four harvests were taken, and three others in which there were three harvests. In the latter experiments another aspect of the problem was under simultaneous investigation (see next section) so that not all the plant cultures were available for the simple rate tests.

Exp. 1. This was made during the early autumn months, and the natural illumination was supplemented by artificial light. NH_4NO_3 was used as the source of combined N. The assimilation of N is shown in Fig. 1. From these data it is evident that the inhibition of N fixation by H_2 does not arise from mere restriction of fixation during the early stages of treatment but is an effect which persists throughout the experiment. In contrast to this effect of H_2 on the assimilation of elemental N the uptake of combined N is independent of the pH_2 in the atmosphere.

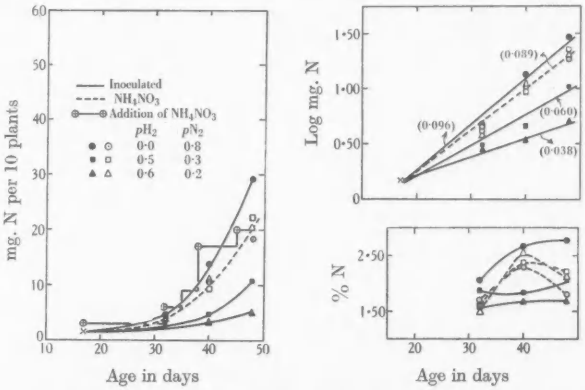

Fig. 1. Comparison of assimilation of free and combined N (NH_4NO_3) by red clover in atmospheres of various pH_2. Figures in parentheses represent g values.

(Exp. 1, planted 12 September; all cultures in air until harvest I, 29 September, then changed to atmosphere indicated; harvest II, 14 October; harvest III, 22 October; harvest IV, 30 October, 1936.)

When log mg. N is plotted against time, the fit of the points to a straight line is quite satisfactory except possibly of those for the atmosphere containing a pH_2 of 0·4 atm. Even in this case the deviations from the line hardly exceed sampling errors. The points corresponding to the plants given combined N fall along a single line, so the g values are identical for these plants whether H_2 is present or not. In contrast, the g values for plants dependent on the fixation of free N decrease with an increase in the pH_2 of the atmosphere.

The effect of H_2 on the % N in the plants should not be overlooked. With *inoculated plants* there is a marked decrease in the % N with increase in the pH_2 of the atmosphere. This means that N fixation is so restricted in these plants when grown in the presence of H_2 that carbohydrates accumulate. The appearance of the plants reflects this difference in composition; plants grown in air are

dark green in colour whereas those grown in an atmosphere containing H_2 have yellow leaves and red stems. With plants given combined N there is no association of % N with the pH_2, and the plants are indistinguishable in general appearance.

It is emphasized that the effect of H_2 on the N fixation process does not arise from the simultaneous decrease in the pN_2 of the atmosphere since in the range used the rate of fixation is independent of pN_2 [Wilson, 1936].

Exp. 2. As this was done during the summer months, light and other weather conditions were ideal for N fixation. The roof of the greenhouse was equipped with a water cooling system, and the bottles were kept in a trough of flowing water in order to avoid excessive temperatures. Under these conditions the development of the plants was most satisfactory. In Exp. 1, through control of the addition of NH_4NO_3, the rate of assimilation of combined N was kept nearly equal to the rate of assimilation of free N_2 by the inoculated plants. The objection could be raised that such a procedure might mask an inhibition by H_2 of the uptake of combined N. If some other factor, such as supply of combined N, is restricting the growth of the plants, inhibition by H_2 might not be obvious. In Exp. 2 $(NH_4)_2HPO_4$-N was always supplied in excess so that the rate of uptake was not restricted because of low concentration of available combined N. $(NH_4)_2HPO_4$ was used, as other experiments had indicated that development of clover was less erratic with this form of N [Wilson & Umbreit, 1937].

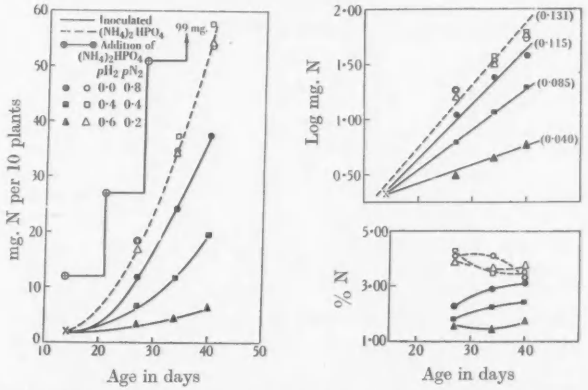

Fig. 2. Effect of pH_2 in atmosphere on assimilation of free and combined N ($(NH_4)_2HPO_4$) by red clover.

(Exp. 2, planted 6 July 1937; all in air until harvest I, 20 July, then changed to indicated atmospheres; harvest II, 2 August; harvest III, 9 August; harvest IV, 15 August.)

The data given in Fig. 2 confirm in every way the conclusions discussed in connexion with Exp. 1. The fit of the experimental points to the straight lines in the semi-log plotting is very satisfactory. These results point to an unmistakable inhibition of the fixation of free N by H_2, an inhibition which is not evident in the assimilation of combined N.

Exps. 3 and 4. These are a part of the series to be discussed in the next section in which the treatment was changed during the course of the tests. Only the semi-logarithmic plots (Fig. 3) are given since these summarize the results of chief interest. Data concerned with the % N were similar to those given in

Figs. 1 and 2. It will be noted that in these experiments the plants receiving combined N were also inoculated. Unless combined N is maintained at a high level, the plants will obtain part of their supply of this element through fixation. In these particular experiments it was desired to keep the rate of development of the plants in the combined N series close to that of the plants dependent entirely on fixation for their N. This was accomplished, as is shown by the g values given in Fig. 3, but the rate of adding NH_4NO_3-N did not entirely prevent fixation of

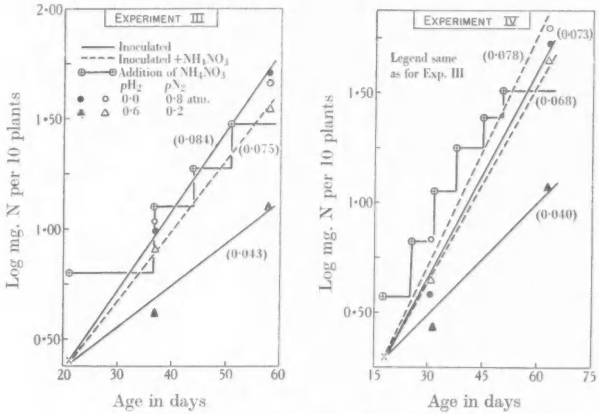

Fig. 3. Effect of H_2 on assimilation of free N_2 and NH_4NO_3 by inoculated red clover plants.

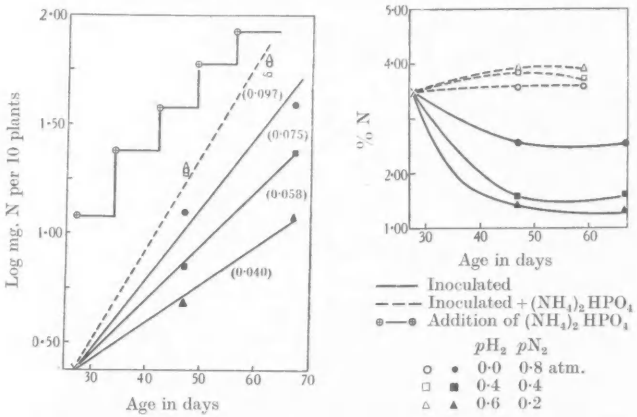

Fig. 4. Effect of presence of H_2 in atmosphere on assimilation of free N_2 and $(NH_4)_2HPO_4$ by red clover.

(Exp. 7, planted 19 August 1937; all kept in air until harvest I, 15 September; then changed to different treatments.)

elemental N by the plants of the combined N series. At harvest, well-developed nodules were found on all the plants. Under such circumstances, it would be expected that the plants of the inoculated plus combined N series which were

grown in the atmosphere containing H_2 would show some evidence of inhibition. As is shown in Fig. 3, such evidence was obtained; the g values for the plants supplied with combined N and kept in an atmosphere with a pH_2 of 0·6 atm. were slightly but significantly lower than those of similar plants kept in air.

Exp. 7. In this experiment the combined N, $(NH_4)_2HPO_4$, was added at such a rate that it was always in excess. As a result fixation of atmospheric N_2 by the plants of the inoculated plus combined N series was entirely suppressed, and the slight inhibition in the development of the plants because of presence of H_2 observed in Exps. 3 and 4 was not obtained (Fig. 4). Plants dependent entirely on fixation for their N showed the "hydrogen effect" both in rate of fixation and in % N in the plants.

The results of these five experiments concerned with the effect of H_2 on the symbiotic N fixation process in red clover consistently support the conclusion that inhibition of N fixation by H_2 persists throughout the growth of the plant and is not merely an initial effect associated with placing the plants in an atmosphere to which they are unaccustomed. The extents of inhibition by a pH_2 of 0·6 atm. as measured by the values of g were as follows: 57, 65, 49, 45 and 47 %. If the total quantity of N fixed is used as the criterion, inhibition reaches 65 to 75% [Wilson & Umbreit, 1937]. The maximum inhibition was observed when the rate of development of the plant was greatest. If environmental conditions are not optimum for growth, fixation is restricted by other factors, e.g. a low supply of carbohydrate, and the extent of inhibition by H_2 is partially masked. Irrespective of the rate of growth of plants in air, the observed g values for plants kept in an atmosphere containing a pH_2 of 0·6 atm. were about 0·040. This means that H_2 was restricting fixation to such an extent that other factors which decrease fixation were relatively ineffective.

The most effective method for decreasing the growth rate of plants kept in air is to reduce the supply of carbohydrate, e.g. by shading. Such treatment would scarcely affect N fixation by plants grown in presence of H_2 since inhibition of fixation by this gas favours the accumulation of carbohydrate, an effect which would compensate for the reduced photosynthesis. The lowest value of g for plants grown in air for which clear-cut inhibition could be readily demonstrated would therefore be in the neighbourhood of 0·050–0·060.

In this connexion it appears that the range of growth rates which may be covered in this type of work is limited. In spite of growing the cultures under widely differing environmental conditions, especially with respect to intensity and duration of light, the g values for inoculated plants kept in the air varied only from 0·073 to 0·115. The g value of 0·115 was observed in an experiment in which the environment was close to ideal for maximum fixation of N. In this same experiment plants supplied with an excess of $(NH_4)_2HPO_4$ assimilated combined N at a rate equivalent to a g value of 0·131. It appears doubtful, then, whether a g value considerably higher than 0·115 could be obtained for fixation of N under the experimental conditions employed.

Change of treatment experiments

The purpose of these experiments was to show that inhibition of N fixation by H_2 was readily reversible and was not associated with any definite stage or rate of growth. In order to demonstrate reversibility of the inhibition, inoculated red clover plants were grown in an atmosphere containing H_2 until inhibition was evident. They were then either changed to an atmosphere free of H_2 or were kept in the H_2-containing atmosphere but supplied with combined N. That the

inhibition was not associated with any particular stage or rate of growth was shown by growing inoculated clover plants in air, then transferring to an atmosphere containing H_2 after fixation had proceeded for various periods of time. Occasionally, in these experiments one of the duplicate cultures of the combined N series would have to be discarded because of chance infection with algae.

Exps. 3 and 4. The data from these experiments are summarized in Table I; briefly, they show that:

Table I. *Effect of change in atmosphere supplied to inoculated red clover plants on assimilation of free N_2 and NH_4NO_3 (Exps. 3 and 4)*

Harvest I to II. Data for harvest II					Harvest II to III. Data for harvest III				
Atmosphere	NH_4NO_3	Total N* mg.	% N	g†	Atmosphere	NH_4NO_3	Total N* mg.	% N	g
Exp. 3:									
Air	–	9·8	2·80	0·084	Hydrogen‡	–	17·9	1·52	0·031
							19·6	1·48	0·035
Air	+	10·9	2·67	0·084	Air	–	47·1	2·95	0·077
							45·6	2·54	0·076
					Hydrogen	–	16·2	1·45	0·028
							22·9	1·52	0·044
Hydrogen	–	4·1	1·60	0·043	Air	–	29·8	2·70	0·087
							26·2	2·67	0·082
					Hydrogen	+	30·0	2·50	0·088
							—	—	—
Hydrogen	+	8·3	1·91	0·075	Air	–	30·7	2·59	0·065
							52·8	3·00	0·090
					Hydrogen	–	18·8	1·55	0·041
							20·5	2·05	0·045
Exp. 4:									
Air	–	4·1	2·58	0·073	Hydrogen	–	19·3	2·07	0·043
		3·6	2·10				19·3	1·69	0·043
Air	+	7·2	3·00	0·078	Air	–	51·8	2·70	0·070
		6·5	2·46				58·8	2·68	0·074
					Hydrogen	–	25·8	1·85	0·047
							25·4	1·80	0·046
Hydrogen	–	2·72	2·08	0·040	Air	–	41·6	3·03	0·081
		2·18	1·58				32·2	2·42	0·073
					Hydrogen	+	22·3	2·63	0·061
							—	—	—
Hydrogen	+	3·8	2·31	0·068	Air	–	60·5	2·81	0·081
		5·1	2·17				66·5	2·87	0·083
					Hydrogen	–	15·7	1·97	0·039
							—	—	—

	Exp. 3	*Exp. 4*
Planted	3 March 1936	2 November 1936
Harvest I	8 April	20 November
II	24 April	3 December
III	15 May	4 January 1937

* Per 10 plants.

† These g values are taken from the lines of Fig. 3; they represent those for the cultures in which the treatments indicated in columns 1 and 2 were unchanged throughout the experiment. Similarly, the g values for harvest II! are calculated from the points on these lines at harvest II, rather than the actual experimental points.

‡ Hydrogen = pH_2, 0·6 atm.; pN_2, 0·2 atm.; pO_2, 0·2 atm.

All cultures were inoculated and kept in air until harvest I, then changed to treatments indicated in columns 1 and 2, until harvest II, and finally changed to treatments indicated in columns 6 and 7.

(1) If inoculated clover plants are grown in air and then transferred to an atmosphere containing 0·6 atm. H_2, the rate of N fixation decreases from the high value characteristic of plants kept in air to that of plants which have been continuously maintained in an atmosphere of this pH_2.

(2) If clover plants which have been both inoculated and supplied with combined N are grown in air and after a period have their fixed N supply withdrawn, assimilation of free N proceeds at a rate characteristic of plants fixing N in air during the entire experiment. The slight decrease in the observed value of g probably arises from a lag in the development of the nodules occasioned by the presence of combined N in the early stages of development. If the plants are transferred to an atmosphere containing a pH_2 of 0·6 atm. when the supply of fixed N is withdrawn, fixation of elemental N is inhibited so that the g value with such plants drops to one equal to, or perhaps slightly higher than, that characteristic of inoculated plants given no combined N and kept in the H_2-containing atmosphere throughout the experiment. The slightly higher values occasionally encountered are most likely to be ascribed to residual combined N remaining in the substrate when the transfer is made.

(3) If inoculated plants which have been grown in an atmosphere containing H_2 on a substrate free of combined N are transferred to air, the rate of fixation increases from a low value to one that is typical of plants continuously kept in air. Moreover, if the plants are kept in the H_2-containing atmosphere but supplied with combined N, there is an immediate increase in the rate of assimilation to a value characteristic of plants supplied with combined N throughout the experiment.

(4) If inoculated clover plants are supplied with combined N and grown in an atmosphere with a pH_2 of 0·6 atm., the rate of N assimilation is only slightly less than that of similar plants grown in air (see discussion of Exps. 3 and 4 in the preceding section). If the supply of combined N is withdrawn, and the plants are kept in the same atmosphere, the rate of assimilation of free N assumes a value characteristic of inoculated plants grown in the presence of this pH_2. If, however, at the time of withdrawal of the combined N the plants are transferred to air, assimilation of free N proceeds at a rate typical of inoculated plants grown in air.

In all these various responses corresponding changes in the % N in the plants occurred coincidently with the changes in the rate of assimilation of N. It is clear from these results that the inhibition of N fixation by molecular H_2 is independent of the previous growth history of the plant and that the effect is readily reversible, that is, transfer from a H_2-containing atmosphere to air results in an increase in the rate of fixation and *vice versa*.

Exp. 5. In this experiment all plants were inoculated and kept in air until N fixation had begun, when all were placed in an atmosphere with a pH_2 of 0·6 atm. When inhibition of fixation had proceeded to the stage where the plants turn yellow because of excessive carbohydrate, four series of different treatments were formed. Plants of *Series 1* were transferred to air; those of *Series 2* were kept in the atmosphere containing H_2, but an excess of combined N as $(NH_4)_2HPO_4$ was added; plants of *Series 3* were treated as those of *Series 2* except that the combined N was supplied as $Ca(NO_3)_2$; plants of *Series 4* were kept in the H_2 atmosphere with no treatment. The essential data from this experiment which are shown in Fig. 5 provide additional evidence that the action of H_2 in the atmosphere is definitely concerned with the N fixation process and not with the general growth of the plant. If plants are returned to air after inhibition of fixation by H_2, the rate of N fixation soon reaches a value characteristic of fixation in air. This demonstrates that the effect of the H_2 is reversible. It is unnecessary, however, to return the plants to air in order to bring about normal development as this also can be accomplished by supplying combined N to the plants which are maintained in the atmosphere containing H_2. Coincidently with the increase in rate of fixation (or assimilation of combined N) after change in treatment there occurs an increase in the percentage N in the plants.

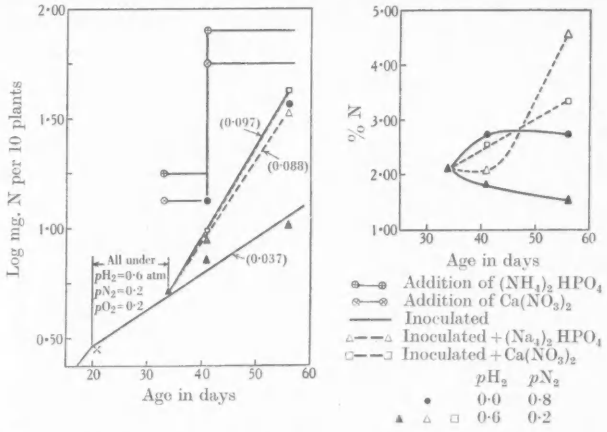

Fig. 5. Effect of change in treatment on assimilation of free and combined N by red clover.

(Exp. 5, planted 2 February 1937; all kept in air until harvest I, 19 March, then placed in H_2-containing atmosphere; treatments changed as indicated at harvest II, 2 April; harvest III, 19 April; harvest IV, 24 April.)

Table II. *Effect of source of N on inoculated red clover plants in presence and absence of H_2 (Exp. 6)*

Atmosphere	Source of N	Total N mg.	% N	*g*	Addition of combined N
Exp. 6:					
Air	N_2	30·9	3·12	0·152	2 Aug. —24·2 mg. NH_4^+–N
		30·9	2·96	0·152	23·9 mg. NO_3^-–N
	$Ca(NO_3)_2$	16·5	3·62	0·108*	
		27·6	3·61	0·144	
	$(NH_4)_2HPO_4$	42·8	3·44	0·176	10 Aug.—24·2 mg. NH_4^+–N
		32·9	3·81	0·157	23·9 mg. NO_3^-–N
pH_2=0·6 atm.	N_2	5·34	1·73	0·028	
pN_2=0·2 atm.		7·18	1·84	0·049	
pO_2=0·2 atm.		4·91	1·60	0·022	
	$Ca(NO_3)_2$	8·06	2·18	0·057*	
		33·8	3·51	0·157	
	$(NH_4)_2HPO_4$	26·0	3·45	0·140	
		28·5	3·57	0·147	

* See text for discussion of these values.

All cultures were inoculated and kept in air until harvest I; all changed to an atmosphere of pH_2=0·6, pN_2=0·2 and pO_2=0·2 atm. until harvest II; then to treatments indicated in columns 1 and 2 of the table.

Planted	6 July 1937
Harvest I	20 July (2·12)
II	2 August (3·63)
III	16 August

Figures in parentheses refer to nitrogen content of 10 plants at indicated harvest.

This experiment was made simultaneously with Exp. 2 (Fig. 2). The *g* values for cultures kept in air throughout experiment were:

Inoculated, 0·115;
Inoculated plus $(NH_4)_2HPO_4$, 0·131.

Those for cultures kept in pH_2 0·6 atm. were:

Inoculated, 0·040;
Inoculated plus $(NH_4)_2HPO_4$, 0·131.

Exp. 6. This is of chief interest as it illustrates a type of variation in response to change in treatment which is occasionally encountered, viz. delay in the recovery of plants on addition of combined N after these have been kept under a rather high pH_2 for some time. The experiment, the general plan of which was identical with that of Exp. 5, was conducted during the summer under conditions which favoured a high rate of photosynthesis. As a result the plants which had been kept in the H_2-containing atmosphere developed an excessive carbohydrate-nitrogen balance as evidenced by yellow leaves and red stems. Inoculated plants transferred to air responded immediately to the change in treatment, and in a few days the colour of the plants was changing to green. N fixation was evidently stimulated by the excessive carbohydrate [Wilson, 1935] since the value for g, 0·152, was the highest obtained in these experiments for assimilation of free N_2 (see Table II). The response of the plants given an excess of combined N (which would effectually prevent fixation of the element) was somewhat erratic. Uptake of the combined forms started immediately in the majority of the plants, but two cultures which were furnished $Ca(NO_3)_2$ did not begin assimilation for several days as judged by appearance of green colour in leaves and stems. After 7–10 days, assimilation appeared to take place normally, but the initial lag caused the plants to contain less N at harvest than did the others so that the calculated values of g for these two cultures were definitely lower.

Exp. 7. In this experiment further evidence of the occurrence of a lag in uptake of combined N by red clover plants previously kept in an atmosphere

Table III. *Effect of change in the atmosphere on assimilation of free and combined N (Exp. 7)*

	Harvest I to II			Harvest II to IV		Data of harvest III			Data of harvest IV		
Treatment	Total N mg.	% N	g*	Atmosphere	Source of N	Total N mg.	% N	g	Total N mg.	% N	g
Exp. 7:											
Air	13·1	2·64	0·075	Hydrogen†	N_2	21·4	1·90	0·047	—	—	—
						18·7	1·75	0·038	24·3	1·76	0·029
pH_2=0·6 atm.	4·63	1·46	0·040	Air	N_2	24·0	3·03	0·103	—	—	—
pN_2=0·2 atm.						22·0	3·14	0·097	38·8	2·75	0·075
pO_2=0·2 atm.				Air	NH_4	—	—	—	62·9	3·55	0·105
						30·3	3·93	0·118	44·0	3·76	0·054
				Air	NO_3	19·6	2·94	0·089	36·2	3·00	0·051
						31·2	3·43	0·120	40·0	3·49	0·065
				Hydrogen	NH_4	24·2	3·37	0·103	56·8	3·50	0·131
						20·8	2·79	0·093	42·4	3·52	0·091
				Hydrogen	NO_3	34·6	3·94	0·127	—	—	—
						25·2	3·29	0·106	46·8	3·41	0·064

* These values of g are for cultures kept in atmosphere indicated in column 1 throughout experiment (see Fig. 4).

† Hydrogen = pH_2, 0·6 atm.; pN_2, 0·2 atm.; pO_2, 0·2 atm.

All cultures planted and inoculated—19 August 1937.

Harvest I Placed under atmospheres given in column 1—15 September.
II Changed to treatment given in columns 5 and 6—5 October.
III 20 October.
IV 27 October.

Addition of combined N:

	$Ca(NO_3)_2$	$(NH_4)_2HPO_4$
6 October	24·2 mg.	24·2 mg.
12	24·2 mg.	24·2 mg.
20	24·2 mg.	24·2 mg.

containing H_2 was obtained. Two harvests were made following the change of treatment, but since the rates of fixation were not always constant, values of g were calculated for each harvest. The results, summarized in Table III, confirm those of the previous experiments. If inoculated red clover plants which have been grown in air until N fixation is actively under way are transferred to an atmosphere containing a pH_2 of 0·6 atm., there is an immediate decrease in the rate of fixation. On the other hand, if the plants are first kept in the H_2-containing atmosphere until a carbohydrate excess accumulates, and then placed in air, decided acceleration in the rate of N fixation occurs. But if such plants are furnished with sufficient combined N to stop fixation of the free element, uptake of the combined forms may be delayed for several days. After a lag period of 7 to 10 days assimilation of combined N begins, and the plants develop rapidly. They do not, however, succeed in overtaking those cultures which begin the assimilation of combined N immediately after its addition.

The cause of this unexpected lag in the uptake of combined N in some of the plant cultures is unknown, but the following characteristics of its occurrence should be noted.

(1) The lag is observed only in plants which have an excess of carbohydrate as a result of being placed in an atmosphere which restricts fixation of elemental N_2. In some respects its occurrence is the reverse of that previously noted by Fred *et al.* [1938] who found that soy beans high in carbohydrates began assimilation of free N_2 only after addition of small quantities of combined forms of this element.

(2) It is noteworthy that fixation of elemental N_2 by these clover plants was unaffected by the previous growth in the H_2-containing atmosphere. This difference in the assimilation of free and combined N might be used as support for the view of Virtanen & v. Hausen [1931] that the element is a superior source of N for nutrition of the clover plant.

(3) The lag period is not a typical response since, with the majority of the cultures, uptake of combined N occurred almost immediately after its addition, and certainly as soon as fixation of the free element could be detected in the inoculated cultures transferred to air.

(4) The delay in assimilation of combined N does not appear to be associated with the presence of H_2 since some of the plants transferred to air likewise exhibited the lag period.

Discussion

The experiments reported in this paper together with those previously discussed [Wilson & Umbreit, 1937] provide four types of evidence for the view that H_2 is a specific inhibitor of N fixation by the symbiotic system in red clover. These are:

1. The total quantity of N fixed by inoculated red clover plants is linearly dependent on the pH_2 in the atmosphere, whereas the assimilation of combined N is independent of the pH_2 within experimental error.

2. Clover plants which have been grown in atmospheres of different partial pressures of H_2 show significant differences in the total quantity of elemental N_2 fixed, but similar plants supplied with combined N do not assimilate significantly different quantities of the combined forms.

3. The rates of assimilation of both free and combined N are essentially logarithmic during the period of growth under study which allows calculation of the unimolecular constant of N uptake—the so-called g value. Values of g are significantly different for plants grown in atmospheres of differing pH_2 only if the plants must use free N_2 for their source of this element.

4. Inhibition by H_2 is obtained at different stages of growth and is reversible. For example, inoculated clover plants transferred from an atmosphere containing H_2 to air immediately show an increase in the rate of fixation. Moreover, if the

plants are not transferred but supplied instead with combined forms of the element, uptake of the combined N and development of plants proceed at rates which are strictly comparable with those of similar plants kept in air.

Seventeen experiments have been made during 5 years with consequent fairly large variations in the environmental factors which will influence assimilation of either free or combined N, e.g. intensity and duration of light, temperature etc. The results of all the experiments consistently point to the view that the action of H_2 on the development of inoculated red clover plants is specifically associated with N fixation. All attempts to detect a significant effect on the general development of the plant apart from the fixation of free N_2 or to correlate the action with any particular stage or rate of growth were unsuccessful. On the basis of these data, it is therefore concluded that H_2 is a specific inhibitor for the symbiotic N-fixing system in red clover.

Summary

Additional evidence has been furnished which confirms the conclusion reached from results of previous work: molecular H_2 is a specific inhibitor for the symbiotic N fixation process in inoculated red clover plants.

The assimilation of both free and combined N by red clover under the conditions of the reported experiments is sufficiently close to logarithmic to allow calculation of the unimolecular constants of N assimilation, the g values. These constants are particularly useful for detection of stimulating or inhibiting effects of a substance on the rate of a given reaction. Values of g for assimilation of free N_2 were significantly decreased through addition of H_2 to the atmosphere, but those for assimilation of combined N were independent of the presence of H_2 in the atmosphere.

That the inhibitory action of H_2 is not associated with any particular stage or rate of growth was demonstrated by transferring plants to an atmosphere containing H_2 after growing in air for various periods of time. It was also shown that the action was reversible since plants kept in the presence of H_2 immediately increased their rate of N assimilation if transferred to air or if combined N was supplied to them.

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CCLXX. THE ORIGIN AND SIGNIFICANCE OF SALIVARY PHOSPHATASE

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Part I. The origin of salivary phosphatase

The occurrence of a phosphatase in saliva is generally accepted. Demuth [1925] appears to have been the first to study the activity of this enzyme in saliva. Adamson [1929] and Smith [1930] made important advances in this subject. Both observers considered the phosphatase activity of saliva to be related to the formation of calculus (tartar) on the teeth.

It is desirable to consider the origin of salivary phosphatase. Adamson [1929] found that tissue removed from the gums showed phosphatase activity and that saliva contained a substrate for this enzyme, since on incubating saliva and this tissue (or bone phosphatase) there was an increase in free phosphate. He definitely associated the occurrence of these two factors with the formation of calculus on teeth, a process he was able to imitate *in vitro*. Smith [1930] followed up Adamson's work and found in saliva a phosphatase which he believed to originate from epithelial scales, because centrifuged saliva had no phosphatase activity. He found that this enzyme had an optimum *p*H of 5·5 and could hydrolyse the naturally occurring phosphoric esters of saliva as well as the Na glycerophosphate. He also found that if tissue were removed from the gums and placed in Ca glycerophosphate, a deposition of Ca phosphate occurred in the epithelium.

Since phosphatases are widely distributed in living matter and are found in many animal tissues, organs and secretions, their presence in saliva was readily accepted. Umeno [1931], however, found that neither saliva nor gastric juice contained any phosphatase, while both salivary glands and gastric mucosa showed phosphatase activity, although much less than kidney and duodenal mucosa. It occurred to us that since saliva contains a large variety of micro-organisms, these might be responsible, in part at least, for the phosphatase activity. Evidence is gradually accumulating that phosphatases occur commonly and perhaps generally in micro-organisms. Their occurrence in yeast juice is classical knowledge. The comparison of yeast and muscle metabolisms led to the association of phosphatases with carbohydrate breakdown. The phosphatases of takadiastase from *Aspergillus oryzae* have been studied systematically by Akamatzu and his colleagues, an account of their work being given in a review by Folley & Kay [1936]. Gordon & Cooper [1931; 1932] appear to have made the first observations on bacterial phosphatases. Heard & Wynne [1933] and Pett & Wynne [1933] studied the occurrence and properties of several types of bacteria of widely different cultural characteristics. Wiggert & Werkman [1938] remark that there is now considerable evidence for the importance of phosphorylation in the metabolism of micro-organisms.

We were able to demonstrate phosphatase activity in some micro-organisms, which are commonly air-borne and are therefore likely to be present in the mouth, and in two strains of actinomyces which are of special interest in the

present work, one freshly isolated from the human mouth. The full details of this part of the work will be given later.

To investigate the question of the origin of the salivary phosphatase, we set out to test the activity of cell-free and bacteria-free saliva. The phosphatase activity was tested in the following way, using 15 ml. graduated tubes.

Tubes 1 and 2. 10 ml. substrate + 5 ml. buffer *p*H 5·5 + 1 ml. chloroform water + 0·5 ml. saliva. Mix and add 2 ml. glacial acetic acid and put on ice. These are controls.

Tubes 3 and 4. As above, but incubate a suitable time before the addition of acid.

The volumes were all made up to 15 ml., filtered and the free phosphate estimated by the method of Fiske & Subbarow [1925]. Glacial acetic acid gives with saliva a much clearer filtrate than the more usually employed trichloroacetic acid, and in this way we avoided, in part, the difficulty of turbidity encountered by Smith. The excess of free phosphate in tubes 3 and 4 over that in tubes 1 and 2 gave the phosphatase activity of the saliva.

We compared the activities of native and centrifuged saliva (centrifuged at 2000 r.p.m. for 20 min.) and although the latter had less activity, it was by no means devoid of activity as Smith [1930] stated. Giri [1936] centrifuged saliva, in the process of purification of the phosphatase, without apparent loss of the enzyme. He was not, however, concerned with the origin of this enzyme. Saliva, filtered through nos. 30 and 42 Whatman filter papers, which keep back epithelial scales, also possessed phosphatase activity and, as expected, showed the presence of micro-organisms on culture and incubation in broth at 37° for 24 hr. Three samples of saliva which had been put through a Seitz filter (disk EK) and one which had been through a Berkefeld N filter were also investigated. The Seitz-filtered sample proved sterile, on testing by seeding into broth and incubating at 37°. The Berkefeld sample was not sterile.

These results are represented in Table I. It is to be noted that salivas show very different degrees of phosphatase activity and occasionally no activity. This, in itself, may be significant.

Table I. *Phosphatase activity of saliva. Hydrolysis of Na α-glycerophosphate at* p*H 5·5*

Sample	Native	Centrifuged	Filtered	Remarks
1	+ +	+	O	Seitz EK sample had no ptyalin and no Ca or P. Sterile
2	+ +	+	O	Ditto
3	O	–	–	Not pooled. Ptyalin present
4	+ +	+	S	Berkefeld. Not sterile
5	+ +	+	O	Seitz as above
6	O	–	–	Not pooled. Ptyalin present
7	+ +	+	–	

(S = slight activity; O = no activity; +, + + = activity of different degrees; – = not tested.)

In three of the experiments, the relative activities of native and centrifuged salivas were calculated, with the following results:

Saliva sample no.	4	5	7
$\frac{\text{Activity native}}{\text{Activity centrifuged}}$	1·4	1·4	3·1

The actual values obtained for sample 7 were:

Native saliva ... ... 30·0 mg. P set free in 18 hr. per 100 ml. saliva
Centrifuged saliva ... 9·5 mg. P set free in 18 hr. per 100 ml. saliva

In considering the filtered salivas, although the Seitz-filtered sterile saliva had no phosphatase activity and the Berkefeld-filtered non-sterile sample did possess activity, we cannot say, without reservation, that the activity of saliva is normally due to these organisms. An interesting observation was made in these saliva experiments. The Seitz-filtered saliva (a sample of the original had been shown to have all the normal constituents) contained no phosphatase and incidentally no ptyalin (amylase) and no inorganic P. The Berkefeld-filtered saliva had all the normal constituents.

Presuming that cannulated saliva collected under sterile conditions might be free from micro-organisms, two experiments were done on cats under chloralose.[1] The submaxillary duct was cannulated with a sterile cannula. Pilocarpine was injected intravenously to excite secretion. This duct secretion and the saliva from the mouth were tested for phosphatase activity. The results are given in Table II.

Table II. *Phosphatase activity of saliva*

	Substrate		
	Na α-glycero-phosphate 18 hr.	Di-Na-phenyl phosphate ½ hr.	Remarks
Cat I:			
Duct secretion	S	O	Saliva very watery. Sterility not tested
Saliva from mouth	+ + +	O	Very viscid
Cat II:			
Duct secretion	S	O	Very watery. No cells present. Few bacteria in tubes incubated 18 hr.
Same duct secretion, mouth scrapings added	+ +	–	Scrapings from roof of mouth added
Saliva from mouth	+ + + + + +	+ +	Viscid. Cells and many organisms present

Note. The activity of saliva towards phenyl phosphate is always less than towards glycerophosphate, probably because at the optimum *p*H for salivary phosphatase, the phenyl phosphate does not hydrolyse well.

It is possible that more rigidly sterile conditions than we were able to obtain would give a secretion free of phosphatase. We do not think that the slight activity of the duct secretion was due to the presence of cells. Another possibility is that the other salivary or the buccal glands might produce the phosphatase.

If the desquamated cells in the saliva are not the sole source of the phosphatase, and it is thought that micro-organisms contribute to the activity, then clearly another way of investigating this latter possibility is to compare the phosphatase activity of saliva straight from the mouth, as uncontaminated with airborne organisms as possible, with the activity of the same saliva incubated in a nutrient medium for a suitable time at 37°.

A series of such experiments was carried out, the saliva being tested as described previously at *p*H 6 using glycine buffer and glycerophosphate. The details of the conditions of these experiments and the results are set out in Table III.

There can be no doubt that saliva contains micro-organisms whose multiplication is accompanied by an increase in phosphatase activity. It may be noted here that Smith [1930] found that saliva had less activity after brushing

[1] These experiments were performed at Bedford College by M. M. Murray.

Table III. *Phosphatase activity of saliva after incubation*

Details of incubation	Time of incubation in days	Phosphatase activity in mg. P/100 ml. saliva: α-glycero-phosphate	Phosphatase activity in mg. P/100 ml. saliva: β-glycero-phosphate
Saliva and Czapek-Dox (KH_2PO_4 omitted)	0	1·0	1·0
	1	1·0	1·0
	5	44	34
Saliva and sterile nutrient broth	0	4·6	—
	2	67	—
Saliva and sterile glucose broth	0	2·5	—
	3	58	—
The same in presence of 0·7% T.C.P.	3	6·6	—

the teeth, a process which certainly diminishes the vegetative growth which forms part of dental "plaque". The conclusion we have arrived at from the experiments on centrifuged saliva, submaxillary duct saliva of the cat and the incubated saliva is that the phosphatase of saliva could originate, partly at least, from the micro-organisms present.

Part II. The relation of calculus (tartar) formation to micro-organisms and salivary phosphatase

The work of Adamson [1929] and that of Smith [1930] lead to the same general conclusions, namely, that the deposition of Ca phosphate and hence also of calculus was brought about by the activity of the phosphatases in either gum tissue or saliva. Tenenbaum & Karshan [1937] found significantly higher Ca and P values in saliva from mouths where calculus formation was greater.

Calculus formation has been correlated with the occurrence of certain micro-organisms, viz. *Leptothrix* (now called *Actinomyces*) by several investigators and some of these workers, i.e. Goodrich & Moseley [1916] and Siegel [1935] relied on histological methods. Naeslund [1926] and Bibby [1935] used both cultural and histological methods, while Bulleid [1925] carried out experiments both *in vitro* and on cats from which he deduced that calculus formation was related to the occurrence of *Leptothrix*. It may be pointed out that Henrici [1930] cast doubt on the value of examination and identification of this type of organism when carried out solely by microscopical examination of stained preparations. This criticism of Henrici gains weight when the results just mentioned are contrasted with those of Lord & Trevett [1936] who obtained four cultures of *Actinomyces* from 90 mouths and Erikson [1937] who was only once successful in growing *Actinomyces* from teeth, although a number of attempts were made and the investigator had grown 50 strains of *Actinomyces* from various sources over a period of years [Erikson, 1935]. Attempts by one of us (P. P.) to isolate *Actinomyces* were at first unsuccessful even from a case of true clinical actinomycosis. Later in the progress of the work an aerobic *Actinomyces*, identical with those placed in group I by Erikson (after Ørskov), was isolated in pure culture from the mouth and this strain and also *A. buccalis* (Winslow), which had been maintained in a series of sub-cultures, were included in the study of the phosphatases of certain mouth micro-organisms. This latter culture was obtained from the Lister Institute (National Collection of Type Cultures) through the courtesy of Dr St John Brooks. It originally came from the American National Type Culture, having in the first place been isolated from the mouth.

Following up the line of thought that the phosphatase of saliva is possibly derived from micro-organisms, we were led to investigate in the early part of this work the phosphatase activities of certain micro-organisms which were considered to be common inhabitants of the mouth. In view of the fact that they are widely distributed in air, soil and food, there is no doubt that they are frequently present in the mouth, although they are not necessarily colonized there. These micro-organisms, originally obtained from a collection of extracted teeth and isolated in pure culture in an unorthodox manner, were first studied. Our attention was accidentally directed to these organisms in the process of separation and collection of enamel protein. Sound (non-carious) human incisor teeth were scraped, the roots cut off and the pulps removed. The crowns were exposed to 10% KOH for periods up to $\frac{1}{2}$ hr. and then brushed vigorously in order to remove the mucin. These crowns were washed well in water and then exposed to a large volume of 2% HCl for about 48 hr. As decalcification proceeded, the enamel protein separated as small flakes and the dentine became soft. The dentine remnants were then picked out, leaving the few mg. of enamel protein in the form of floating fragments which could be collected by centrifuging. Work was then suspended for about 6 weeks, during which time the protein was left covered in 2% acid at room temperature. It was hoped that bacterial growth would be restricted in this way, so that work of a bacteriological nature could be carried out on the protein. The *p*H of the liquid was about 0·7. At the end of the period mentioned, it was noted that some "fluffy" growth was attached to the protein. The sources of nitrogen available to the growth were very limited. It was thought at one time that the organisms were using enamel protein as the source of nitrogen, but although there were some indications that this may be true, we were not successful in showing histologically that these organisms had penetrated the pieces of protein. Further work on this point is in progress. From the "fluffy" mass three organisms were isolated. They have been kept in a series of sub-cultures for about 6 months and are pure strains. The method of isolation described above has most certainly eliminated many of the mouth organisms. We have, however, limited our investigations to some of those from human mouths which could withstand exposure to 2% HCl for 6 weeks, and whose nitrogen requirements were limited to the very small amount of extraneous nitrogen-containing compounds which had not been eliminated.

Description of the organisms studied

The three organisms were originally described as "green", "red" and "white", but they have subsequently been classified as far as possible. The "green" organism is a typical strain of *Penicillium spinulosum* (Thom.). The "red" organism is a species of *Rhodotorula* resembling *Rhodotorula rubra* [Harrison, 1928]. Some difficulty has been met in identifying the "white" organism. It grows aerobically only, colonies on Czapek Dox, serum, agar and glucose-agar being smooth, shiny, raised, slowly spreading and very mucilaginous. In slide cultures the mycelium is non-septate. Spores have not been seen. It is thought that the "white" organism is one of the monilia. (The description of the two strains of *Actinomyces* studied has already been given.)

The method of isolation of the organisms from extracted teeth certainly eliminated many of the mouth organisms, but doubt remained as to whether the organisms studied came from the mouth. The teeth examined were at first collected in water saturated with chloroform in small jars, when some moulds grew on the corks. Later all corks and jars were sterilized by exposure to hot

air at 160° for 1 hr., a method which proved to be satisfactory. In some collections of teeth made in these sterilized jars, penicillia still grew. Mould spores from the air might have contaminated the teeth in the interval of 2–4 hr. between the removal from the mouth and the placing in the jars. This possibility was not investigated.

It is to be noted that the primary object of the study of these organisms was to learn something of their proteolytic activity. Simultaneously, however, partly at the suggestion of Prof. F. C. Wilkinson, the possibility of the occurrence of phosphatase came under consideration.

Study of phosphatase activity

Three esters were used as substrates, namely Na α- and β-glycerophosphates (Boots) and di-Na-phenyl phosphate (B.D.H.), the latter purified to remove free phosphate. Each ester was tested at the characteristic *p*H optima of acid and alkaline phosphatases [Folley & Kay, 1936], i.e. at *p*H 3·8–4·0 and *p*H 9·9, using Sørensen's glycine and HCl and glycine and NaOH buffers [Clark, 1928], and also at *p*H 5·5, which was shown to be the optimum of salivary phosphatase by Smith [1930].

The enzyme preparations were made by scraping the growth (4–7 days old) off a Petri dish (10 cm. diameter) agar culture and grinding with sand and sufficient volume of 0·7 % $MgSO_4$ saturated with chloroform to give enough solution for the tests. This was filtered through cotton-wool. One ml. of such a solution contains the optimal concentration of Mg for phosphatase activation. The substrates were 0·1 % Na α- and β-glycerophosphates and 0·109 % di-Na-phenyl phosphate. The tests and adequate controls were carried out according to the following directions. Twelve tubes were set up for each growth with each substrate, each tube containing 10 ml. of the particular substrate, 5 ml. of one of the buffer solutions and 1 ml. of the growth extract. Half of these tubes served as controls and were left in the refrigerator after the addition of 2 ml. 25 % trichloroacetic acid, to stop any enzymic action. The other tubes were incubated at 37° in the case of the phenyl phosphate for ½ hr. and in the case of the glycerophosphates for 18 hr. After this incubation time, the enzyme activity was stopped in the same way as the controls.

Tubes 1 and 2. 10 ml. substrate, 5 ml. buffer *p*H 4 and 1 ml. extract. Incubated at 37° before the addition of acid.

Tubes 3 and 4. 10 ml. substrate, 5 ml. buffer *p*H 4 and 1 ml. extract. 2 ml. trichloroacetic acid added and tubes put on ice.

Tubes 5 and 6. 10 ml. substrate, 5 ml. buffer *p*H 5·5 and 1 ml. extract. Incubated before addition of acid.

Tubes 7 and 8. 10 ml. substrate, 5 ml. buffer 5·5 and 1 ml. extract. Acid added as above and put on ice.

Tubes 9 and 10. 10 ml. substrate, 5 ml. buffer *p*H 10, 1 ml. extract. Incubated before addition of acid.

Tubes 11 and 12. 10 ml. substrate, 5 ml. buffer *p*H 10, 1 ml. extract. Acid added as above and put on ice.

All the tubes were then brought to room temperature and filtered. 10 ml. of each filtrate were tested for free P by the method of Fiske & Subbarow [1925].

Successive sub-culturings were tested in this way and although the results (Table IV) with each of the cultures are not quantitatively comparable, the results obtained with any one culture at different *p*H in any one series are strictly comparable.

Table IV. *The phosphatase activities of the micro-organisms on different substrates at different* p*H*

Substrate	Na α-glycero-phosphate			Na β-glycero-phosphate			Di-Na-phenyl-phosphate		
*p*H	4	5·5	10	4	5·5	10	4	5·5	10
Monilia (white growth)	O	S	+ +	O	S	+ +	O	+	+ + +
R. rubra (Harrison) (red growth)	O	+	S	O	+ +	+	O	S	+ + +
P. spinulosum (Thom.) (green growth)	+	+ + +	S to O	S to O	+ + +	S	O	+ + + +	+
Mixed white and green	O	+ +	O	O	+ +	O	O	+ +	O
A. buccalis (Winslow)	O	O to S	O	–	–	–	–	–	–
Actinomyces, group 1, Erikson	S	+ + +	S	–	–	–	–	–	–
Saliva	+	+ + +	S	O	+ +	O	O	S	O

In our choice of *p*H values at which to test the enzyme activity, we had in mind the separation of the acid and alkaline phosphatases [Folley & Kay, 1936] and the optimum reaction for the salivary phosphatase towards glycerophosphate found by Smith [1930] to be *p*H 5·5, by Giri [1936] *p*H 5·0 and by us *p*H 6·0.

It can be seen from Table IV that the different growths must have different *p*H optima. We were most interested in those showing greatest activity at *p*H 5·5–6 and hence further investigations were made with the green growth. In spite of the fact that *A. buccalis* (Winslow) was tested in six separate young sub-cultures at *p*H 5·5 and *p*H 10, on three different substrates, we were only able to show very slight phosphatase activity and this not constantly. This result must be contrasted with the marked activity of the *Actinomyces* (group 1) freshly isolated from the human mouth. It is quite possible that *A. buccalis* (Winslow) when first isolated, had greater activity. Henrici [1930] noted that *A. gypsoides*, although actively proteolytic when first isolated, gradually, and eventually completely, lost this power after some months of subculturing.

The tests on *Actinomyces* (group 1) were carried out at *p*H 6 only, since it had been shown to have a broad optimum between *p*H 6 and 7.

It is concluded from these tests that certain mouth organisms, including *Actinomyces*, can exhibit phosphatase activity in the mouth. It is, of course, possible that many other mouth organisms also possess phosphatase activity [Fosdick, 1937]. Incidentally, it may be noted that the caries research group of the North Western University, Chicago [see Hatton, 1938], are of the opinion that caries is possibly the result of a combined action of micro-organisms having phosphatase activity, possibly yeasts, together with acid-producing bacteria. We do not wish to comment on this idea, since we have done no work on this point. If, however, the occurrence of these phosphatases is significant, we consider that they should hydrolyse the ester P of blood and saliva.

The green growth was tested on a neutralized trichloroacetic acid protein-free filtrate of whole blood, rat and human, in a similar manner to that of Byrom & Kay [1928] to show that part of the ester P of blood is hydrolysable by kidney phosphatase. An increase of free P resulted on incubation of the green growth with these blood filtrates. With the human blood, the increase amounted to 15 mg. P per 100 ml. blood, a degree of hydrolysis which suggests that the culture had several types of phosphatase in it.

Wilkinson [1935] put forward histological evidence that calculus deposition could be subgingival where no breakaway of the soft tissues from the teeth had occurred. We remark here that these micro-organisms could use the organic

hydrolysable P of the blood plasma and so increase the concentration of free PO_4, which, together with the Ca of the plasma or saliva could conceivably lead to the deposition of Ca phosphate.

It was also considered necessary to show that the saliva itself contains a substrate for these phosphatases. For this test, centrifuged saliva was steamed for 5 hr. without concentration. It became cloudy, but was used without further treatment as a substrate for the green mould in the following experiment:

Tubes 1 and 2. 5 ml. saliva (steamed), 5 ml. buffer *p*H 5·5, 2 ml. glacial acetic acid. Placed on ice.

Tubes 3 and 4. As tubes 1 and 2, but incubated for 18 hr. at 37° before addition of the acid.

Tubes 5 and 6. 5 ml. water to replace the saliva, otherwise the same as tubes 1 and 2.

Tubes 7 and 8. 5 ml. water to replace saliva, otherwise the same as tubes 3 and 4.

This experiment yielded a positive result. Tubes 3 and 4 had greater free PO_4 content than tubes 1 and 2. The actual estimation of this PO_4 set free from the saliva was 0·93 mg. hydrolysable P per 100 ml. saliva. Smith [1930] found between 0·8 and 2·7 mg. organic P per 100 ml. saliva. We consider we have demonstrated the fact that green mould can use saliva as a substrate. Furthermore, extracts of the green *Penicillium* incubated with a 1% solution of Ca glycerophosphate, led to the deposition of a white precipitate, which was readily soluble in dilute acid and was found to contain both Ca and P.

In showing that common mouth organisms, including *Actinomyces*, contain phosphatases which can use serum or saliva as a substrate, a correlation has been brought about between the observations of Adamson [1929] and Smith [1930] on the one hand, and Naeslund [1926] and Bulleid [1925] and Bibby [1935] on the other, concerning the theories of calculus formation.

Summary

1. Human saliva exhibits variable phosphatase activity which is maximum towards glycerophosphate at *p*H 5·0–6·0.

2. The submaxillary duct secretion of cats had only very slight phosphatase activity as compared with the mouth saliva of the same animals at the same time.

3. The phosphatase activity of human saliva is not entirely due to the epithelial scales.

4. Certain common mouth micro-organisms exhibit marked phosphatase activity.

5. The organisms studied included *Penicillium spinulosum* (Thom), *Rhodotorula rubra* (Harrison), a monilia, *Actinomyces buccalis* (Winslow) and *Actinomyces* (group 1, Erikson).

6. The *Penicillium* was able to use the hydrolysable ester phosphorus of blood and saliva as a substrate.

7. Precipitation of calcium phosphate occurs when calcium glycerophosphate is incubated with the *Penicillium*. This may be analogous to calculus formation.

8. The correlation of phosphatase activity with the presence of micro-organisms, especially *Actinomyces*, is important in relation to the theories of calculus formation which have been advanced previously.

For the identification of the *Penicillium* and the *Actinomyces* (group 1) we are indebted respectively to Mr G. Smith and Miss D. Erikson, both of the London School of Hygiene and Tropical Medicine. We wish to thank the Medical Research Council for grants towards the expenses of this work and for personal grants to two of the workers (P. P. and G. E. G.).

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CCLXXI. ENZYMIC PROTEOLYSIS

III. HYDROLYSIS OF ASPARAGINE, ASPARAGINE PEPTIDES AND ANHYDROGLYCYL-ASPARAGINE

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The experiments to be described in the present paper are complementary to those published in a previous communication [Damodaran & Ananta-Narayanan, 1938] on the formation of ammonia during the enzymic hydrolysis of natural proteins. Bergell & Wulfing [1910], who seem to have been the first to study the deamidizing action of proteolytic enzymes on synthetic substrates, showed that *dl*-leucinamide is hydrolysed by pancreatin, the *l*-compound being more readily decomposed than the *d*-isomeride; negative results were recorded in the case of glycinamide, alaninamide and natural asparagine. According to Levene *et al.* [1926] "filtered intestinal juice" splits glycinamide as readily as alanylalanine though not with the same ease as glycylglycine. Grover & Chibnall [1927] observed the hydrolysis of asparagine by intestinal erepsin as well as by a dipeptidase preparation obtained from germinating barley. The hydrolysis of glycinamide and leucinamide was confirmed by Waldschmidt-Leitz *et al.* [1927] using trypsin-free erepsin prepared by adsorption. Melville [1935] attempted to study the deamidizing action of proteolytic enzymes on γ-glutaminyl peptides, but found that the subtrates were so labile that they were spontaneously decomposed in aqueous solutions at the *p*H required for enzyme action.

In the present investigation we have studied the actions of pepsin, trypsin, erepsin and papain on asparagine, glycinamide, *dl*-leucinamide, glycyl-*l*-asparagine, *d*- and *l*-leucylasparagine, chloroacetyl-*l*-asparagine and anhydroglycylasparagine (2:5-diketopiperazine acetamide). Amide hydrolysis was measured by actual distillation of the ammonia and estimation of the latter by a sensitive micro-method. To ensure that quantitative differences in the rate of splitting were not interpreted to mean qualitative differences in specificity, enzyme action was in all cases allowed to proceed for fairly long periods. Some of the subtrates, particularly glycinamide, glycyl- and leucyl-asparagine, were found to be hydrolysed to a slight extent at the *p*H employed for enzyme action, but rigorous controls were run to determine the exact extent of such spontaneous decomposition. The results are summarized in Table I.

Pepsin and purified trypsin have no action on either the peptide or amide bonds in any of the subtrates examined. Trypsin-free erepsin on the other hand has a definite, though slow, deamidizing action on asparagine, the hydrolysis of the amide group proceeding more rapidly in fact than in glycinamide though less rapidly than in leucinamide. The amide group in asparagine peptides does not appear to be directly hydrolysed; a small amount of ammonia is formed from glycylasparagine and *l*-leucyl-*l*-asparagine, but this must depend on the preliminary hydrolysis of the peptide to give free asparagine as no ammonia liberation takes place from *d*-leucylasparagine in which the peptide bond is not split by erepsin. Of particular interest in connexion with peptide hydrolysis is the finding that in chloroacetylasparagine the peptide bond is split by erepsin in

Table I. *Hydrolysis* %

	Trypsin-free erepsin		Erepsin-free trypsin		Pepsin		Papain-HCN	
	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
l-Asparagine	—	10 15 days	—	0·37 7 days	—	0·43 6 days	—	0·33 9 days
Glycinamide	—	10 10 days	—	Nil	—	2·9 9 days	—	1·45 8 days
dl-Leucinamide	—	53 10 days	—	1·0 7 days	—	Nil	—	11·0 7 days
Glycyl-*l*-asparagine	97 10 days	8 26 days	Nil	Nil	Nil	Nil	Nil	Nil
d- and *l*-Leucyl-*l*-asparagine	17 6 days	2 6 days	,,	,,	,,	,,	,,	,,
d-Leucyl-*l*-asparagine	Nil	Nil	,,	,,	,,	,,	,,	,,
Chloroacetyl-*l*-asparagine	77 10 days	10 10 days	,,	,,	,,	,,	,,	,,
Anhydroglycylasparagine	Nil	Nil	,,	,,	,,	,,	,,	,,

spite of the absence of a free amino group adjacent to it; this is analogous with the hydrolysis by this enzyme of chloroacetylalanine observed by Abderhalden & Ehrenwall [1930]. According to the views on enzyme specificity developed by Waldschmidt-Leitz, Grassmann and collaborators (summaries by Grassmann [1928, 1932, 1936]) trypsin-free erepsin consists of a mixture of aminopolypeptidase and dipeptidase. The observations now recorded can be brought into line with the views on enzyme specificity developed by the Willstätter school only on the assumption that, in addition to di- and aminopoly-peptidases, erepsin also contains asparaginase and other amidases as well as peptidases that act specifically on acyl peptides of the type of chloroacetylasparagine. However, the weight of available evidence is against the acceptance of these views of the mode of action of the proteases as final. On the one hand the identification of some of the proteolytic enzymes with crystalline proteins [Northrop, 1930; Northrop & Kunitz, 1934], the changes in specificity shown to take place on keeping of enzymes purified by adsorption [Abderhalden & Schmitz, 1929; Abderhalden & Ehrenwall, 1931] and the dependence of the range of activity of proteases on physical conditions [Fodor, 1930] make it highly improbable that the enzyme preparations with restricted activity obtained by Waldschmidt-Leitz, Grassmann and others really represent individual enzymes in the natural state and not merely fragments of such. Further, numerous recent studies on synthetic peptides, in particular by Abderhalden and by Bergmann, make it clear that the chemical bases of the specificity relations enunciated by the Willstätter school for their enzyme preparations will have to be considerably modified.

With papain-HCN only a slow deamidation with leucinamide and hardly any with other subtrates was noticed. This appears to be in contradiction with the results obtained previously [Damodaran & Ananta-Narayanan, 1938] which showed that papain digestion led to the formation of considerable amounts of ammonia. It has, however, been recently shown by Bergmann *et al.* [1935] that the amide group in peptide amides in which there is no free amino group in the proximity of the amide group is attacked by papain.

The observation that anhydroglycylasparagine is not hydrolysed either at the peptide or the amide bond by any of the enzymes tried is contrary to the claims

of Japanese workers [cf. Shibata, 1934] who claim to have demonstrated the enzymic hydrolysis of diketopiperazines, and provides further confirmation of the findings of Waldschmidt-Leitz & Gärtner [1936] with diketopiperazine acetic and propionic acids and of Greenstein [1936] with lysylglutamic acid anhydride.

With reference to the problem in protein structure which primarily prompted this investigation, the results obtained, taken in conjunction with previous findings [Damodaran, 1932; Damodaran *et al.* 1932; Melville, 1935; Damodaran & Ananta-Narayanan, 1938], agree with the assumption that the amide groups attached to the dicarboxylic amino-acids are the only important precursors of the ammonia formed in the acid hydrolysis of proteins. In the last paper cited above it was concluded that the ammonia liberated during the digestion of proteins by pepsin, trypsin and erepsin in succession was not primarily connected with enzyme action. If asparagine and glutamine are the only ammonia-yielding complexes in proteins this is readily understandable for the following reasons. Peptides of glutamine formed through its free (γ) carboxyl group are, unlike those formed through the amino group studied by Thierfelder & Cramm [1919], as unstable as glutamine and spontaneously decompose in aqueous solution [Melville, 1935] giving rise to ammonia. This non-enzymic ammonia formation could commence practically at the very beginning of peptic digestion and would increase progressively with the amount of glutamine-containing polypeptides set free. Asparagine peptides on the other hand are quite stable in aqueous solution and also, as shown in this paper, not acted upon by either pepsin, trypsin or erepsin. The deamidizing action which erepsin has been shown to have on *free* asparagine can come into play only when digestion is practically complete and is at the same time too slow to cause any appreciable liberation of ammonia. When it is further considered that the proportion of aspartic acid in proteins is invariably much lower than that of glutamic acid it is obvious that practically the whole of the ammonia formed during digestion has its origin in the secondary non-enzymic decomposition of glutamine peptides and of glutamine.

Experimental

Substrates

Glycinamide hydrochloride and dl-*leucinamide* hydrobromide were prepared according to Bergell & Wulfing [1910].

Glycyl-l-*asparagine*. The purified peptide prepared by the method of Fischer & Koenigs [1904] browned at 215° and gave the reactions described by these authors. N found 22·07 %; calc. for $C_6H_{11}N_3O_4$, 22·22 %. Amino-N (Van Slyke) was 124 % of the theoretical, in keeping with the observed abnormality of glycine and glycyl polypeptides in general [Van Slyke, 1911; Fischer & Koelker, 1910].

Leucyl-l-*asparagine* [Fischer & Koenigs, 1907]. Asparagine was coupled with bromo*iso*hexoylbromide and the resulting compound treated with 75 % ammonia at room temperature for 6 days. Owing to the difference in solubility between the two isomeric bromo*iso*capronylasparagines noted by Fischer & Koenigs, two products were finally obtained, viz. pure *d*-leucyl-*l*-asparagine and a mixture of *d*- and *l*-leucyl-*l*-asparagines in which the compound containing the non-natural *d*-leucyl residue predominated. Attempts to obtain the pure *l*-leucylpeptide in pure condition by fractional crystallization were not successful. $C_{10}H_{19}N_3O_4$, $2H_2O$ requires N, 14·95 %; N, found, 15·2 % (for the *d* form) and 15·12 % (for the mixture of *d*- and *l*-).

Anhydroglycylasparagine. This was prepared according to Fischer & Koenigs [1904].

Enzymes

Erepsin-free trypsin [Waldschmidt-Leitz & Harteneck, 1925], trypsin-free erepsin [Waldschmidt-Leitz & Schäffner, 1926] and B.D.H. pepsin were used. Papain was prepared as previously described [Damodaran & Ananta-Narayanan, 1938].

Buffer solutions

For erepsin studies $M/5$ phosphate buffer at pH 7·8, for trypsin $M/5$ veronal buffer at pH 8·4 and for papain $M/10$ citrate buffer at pH 5 were employed. As the usual NH_4OH/NH_4Cl buffer for tryptic studies could not be used on account of the necessity of making ammonia determinations, experiments were conducted to ascertain the suitability of veronal buffer. It was found that veronal could effectively replace phosphate as well as NH_4OH/NH_4Cl in erepsin and trypsin digests. It has the advantage over the former of not interfering with titrations in alcohol or formalin.

Methods

Weighed amounts of the analytically pure subtrates were dissolved in water, neutralized with a few drops of NaOH and, after the addition of buffer solution and enzyme, made up to give usually $M/10$ solutions. A few drops of toluene were added as antiseptic.

Peptide hydrolysis was determined by the usual alcoholic titration and ammonia liberation according to Parnas & Heller [1924] with the modification that when the ammonia to be determined was small titration was carried out by the iodimetric method of Henriques & Christiansen [1916] using $N/280$ acid.

(1) *Asparagine*

Table II. *Exp. 1. Trypsin-free erepsin on* l-*asparagine*

Digest: $M/10$ asparagine containing 10 ml. of erepsin solution in total volume 25 ml. at pH 7·8 in phosphate buffer.

	Ammonia as ml. $N/280$ acid in 2 ml.				
Time	Digest	Substrate control	Enzyme control	Digest (corrected)	Amide hydrolysis %
0	0·04	Nil	0·04	Nil	Nil
6 hr.	0·20	Nil	0·03	0·17	0·32
18 hr.	0·79	Nil	0·03	0·76	1·36
42 hr.	1·92	0·02	0·06	1·85	3·30
4 days	4·00	0·09	0·08	3·82	6·80
10 days	8·78	0·18	0·08	8·52	15·20
19 days	12·16	0·34	0·13	11·65	20·80

Exp. 2. Erepsin-free trypsin on l-*asparagine.* Digest: $M/10$ asparagine containing 5 ml. of trypsin solution in total volume 25 ml. at pH 8·4 (veronal buffer).

Amide hydrolysis in 7 days, 0·37 %.

Exp. 3. B.D.H. pepsin on l-*asparagine.* Digest: 10 ml. of $M/4$ asparagine +5 ml. pepsin solution in $N/20$ HCl containing 1/20 the weight of dry pepsin calculated on the weight of the subtrate+5 ml. $N/5$ HCl. Total volume 25 ml.

Amide hydrolysis in 6 days, 0·43 %.

Exp. 4. Papain-HCN on l-*asparagine.* Digest: 10 ml. $M/4$ asparagine. 5 ml. papain solution 10 ml. citrate buffer pH 5 in total volume 25 ml. No NH_3 in substrate control.

Amide hydrolysis in 9 days, 0·33 %.

(2) *Glycinamide*

Table III. *Exp. 5. Trypsin-free erepsin on glycinamide*

Digest: *M*/12 glycinamide containing 10 ml. erepsin solution in total volume 25 ml. at *p*H 7·8 in phosphate buffer.

	Ammonia as ml. *N*/280 acid in 1 ml.				
Time	Digest	Substrate control	Enzyme control	Digest (corrected)	Amide hydrolysis %
0	0·16	0·12	0·04	0	0
5 hr.	0·26	0·12	0·04	0·10	0·43
21 hr.	0·55	0·25	0·03	0·27	1·16
46 hr.	0·93	0·35	0·08	0·50	2·15
4 days	1·65	0·59	0·06	1·00	4·29
10 days	3·11	0·70	0·08	2·33	10·00
19 days	4·48	1·69	0·11	2·68	11·50

Exp. 6. Erepsin-free trypsin on glycinamide. Digest: 0·3467 g. glycinamide HCl dissolved in water, neutralized with NaOH and made up to 25 ml. 10 ml. of this solution + 5 ml. veronal buffer at *p*H 8·4 + 5 ml. trypsin solution + water to 25 ml.

Amide hydrolysis in 7 days, nil.

Exp. 7. Pepsin on glycinamide. Digest: 0·1906 g. glycinamide HCl dissolved in sufficient HCl so that the final concentration of the HCl was *N*/20. 5 ml. of this solution removed and made up to 6·5 ml. for substrate control. To the residual 20 ml. 5 ml. pepsin solution in *N*/20 HCl added.

Amide hydrolysis, 1·1 % in 2 days, 1·9 % in 9 days.

Exp. 8. HCN-activated papain on glycinamide. Digest: 0·2241 g. glycinamide HCl + 10 ml. citrate buffer, *p*H 5, to total volume 25 ml. 5 ml. removed for substrate control. Residual 20 ml. + 5 ml. enzyme formed the digest.

Amide hydrolysis, 0·85 % in 65 hr., 1·45 % in 8 days.

(3) dl-*Leucinamide*

Table IV. *Exp. 9. Trypsin-free erepsin on* dl-*leucinamide*

Digest: 0·3517 g. *dl*-leucinamide HBr made up to 25 ml. after neutralization and addition of *M*/5 phosphate buffer *p*H 7·8. 10 ml. of this solution + 5 ml. erepsin made up to 25 ml. with water.

	Ammonia as ml. *N*/280 acid in 2 ml.				
Time	Digest	Substrate control	Enzyme control	Digest (corrected)	Amide hydrolysis %
0–½ hr.	0·41	0·19	0	0·22	1·47
6 hr.	2·05	—	0	1·86	12·45
7 hr.	2·14	—	0	1·95	13·05
14 hr.	3·74	—	0	3·55	23·75
24 hr.	5·54	—	0	5·35	35·81
40 hr.	6·05	0·11	0	5·94	39·76
10 days	8·05	0·14	0	7·91	52·94
19 days	7·99	0·17	0	7·84	52·34

Exp. 10. Erepsin-free trypsin on dl-*leucinamide.* Digest: 0·175 g. *dl*-leucinamide HBr dissolved in water, neutralized and made up to 25 ml. with veronal buffer *p*H 8·4. 5 ml. of this removed for substrate control. The residual 20 ml. + 5 ml. trypsin formed the digest.

Amide hydrolysis in 7 days, 0·70 %.

Exp. 11. Pepsin on dl-*leucinamide.* Digest: 0·236 g. *dl*-leucinamide HBr dissolved in 25 ml. 5 ml. removed for substrate control. Residual 20 ml. + 5 ml. pepsin formed the digest.

Amide hydrolysis in 8 days, nil.

Table V. *Exp. 12. Papain-HCN on* dl-*leucinamide*

Digest: 0·1902 g. *dl*-leucinamide HBr at *p*H 5 containing 5 ml. papain in total volume 25 ml.

	Ammonia as ml. *N*/280 acid in 2 ml.				
Time	Digest	Substrate control	Enzyme control	Digest (corrected)	Amide hydrolysis %
0	2·04	0	2·04	0	0
4 hr.	1·94	0	—	0	—
16 hr.	2·02	0	—	0	—
25 hr.	2·23	0	—	0·19	0·94
51 hr.	2·71	0	—	0·67	3·30
4 days	4·01	0	—	1·97	9·70
7 days	4·25	0	—	2·21	10·90

(*4*) *Glycyl*-l-*asparagine*

Table VI. *Exp. 13. Trypsin-free erepsin on glycyl*-l-*asparagine*

Digest: 0·5906 g. glycyl-*l*-asparagine neutralized and made up to 25 ml. with phosphate buffer of *p*H 7·8; 5 ml. of this solution removed for substrate control. Residual solution treated with 5 ml. of erepsin. The control showed no ammonia.

Time	Ammonia in 1 ml. of digestion as ml. *N*/280 acid	% amide split	ml. *N*/10 KOH for 2 ml. of digest	Increase in titre	% peptide split
0	0·18	0	3·68	0	0
4 hr.	0·22	0·14	4·38	0·7	35·2
13 hr.	0·64	1·64	4·99	1·31	65·6
18½ hr.	0·64	1·64	5·18	1·50	75·2
24 hr.	0·78	2·14	5·28	1·60	80·0
48 hr.	0·90	2·57	5·38	1·70	84·6
96 hr.	0·90	2·57	5·60	1·92	95·9
10 days	1·05	3·11	5·63	1·95	97·5
26 days	2·37	7·82	—	—	—

Exp. 14. Erepsin-free trypsin on glycyl-l-*asparagine.* Digest: 0·5906 g. of the substrate made up to 25 ml. with veronal buffer *p*H 8·4. 5 ml. removed for substrate control. Residual 20 ml. + 5 ml. trypsin formed the digest.

No peptide or amide hydrolysis at the end of 5 days.

Exp. 15. Pepsin on glycyl-l-*asparagine.* Digest: 0·5906 g. of glycyl-*l*-asparagine dissolved in 5 ml. *M*/5 HCl and made up to 25 ml. 5 ml. of this removed for substrate control and diluted to 6·5 ml. with *N*/20 HCl. Residual 20 ml. of the solution + 5 ml. pepsin formed the digest. No ammonia in enzyme controls.

No amide or peptide hydrolysis in 7 days.

Exp. 16. Papain-HCN on glycyl-l-*asparagine.* Digest: 0·463 g. substrate at *p*H 5 in citrate buffer containing 5 ml. papain-HCN solution in total volume 25 ml. No substrate control.

No amide or peptide hydrolysis in 9 days.

(5) d- *and* l-*Leucyl*-l-*asparagines*

Table VII. *Exp. 17. Trypsin-free erepsin on* d- *and* l-*leucyl*-l-*asparagines*

Digest: 1·405 g. of the substrate dissolved in water and neutralized. 10 ml. of erepsin made up to 25 ml. with phosphate buffer *p*H 7·8. Controls showed no change and are not included in the table.

Time	Ammonia as ml. *N*/280 acid in 1 ml. digest	% amide split	ml. *N*/10 KOH for 2 ml. digest	Increase in titre value	% peptide split
0	0·5	0	3·61	—	—
6 hr.	—	0	4·09	0·48	12·0
12 hr.	0·56	0·10	4·16	0·55	13·75
24 hr.	0·54	0·10	4·21	0·60	15·0
36 hr.	0·70	0·36	4·23	0·62	15·5
54 hr.	0·88	0·68	4·27	0·66	16·5
5 days	1·21	1·26	4·28	0·67	16·75
10 days	1·70	2·14	—	—	—

Exp. 18. Erepsin-free trypsin on d- *and* l-*leucyl*-l-*asparagines.* Digest: 0·620 g. of the substrate dissolved in water neutralized and made up to 25 ml. with 10 ml. veronal buffer *p*H 8·4 and 5 ml. trypsin. No controls.

No amide or peptide hydrolysis in 6 days.

Exp. 19. Pepsin on d- *and* l-*leucyl*-l-*asparagines.* Digest: 0·878 g. of substrate dissolved in 25 ml. of water and sufficient *N*/20 HCl to bring to *p*H 1·8. 5 ml. removed for substrate control. Residual solution + 5 ml. pepsin formed the digest. Enzyme control showed no change.

No amide or peptide hydrolysis in 8 days.

Exp. 20. Papain-HCN on d- *and* l-*leucyl*-l-*asparagines.* Digest: 0·502 g. of the peptide dissolved in water and made up to 25 ml. with 5 ml. papain solution and 10 ml. citrate buffer *p*H 5. No substrate control.

No amide or peptide hydrolysis at the end of 7 days.

(6) d-*Leucyl*-l-*asparagine*

Exp. 21. Trypsin-free erepsin on d-*leucyl*-l-*asparagine.* Digest: 1·00 g. of substrate dissolved in water, neutralized, and made up to 25 ml. with sufficient phosphate buffer at *p*H 7·8. 5 ml. removed for substrate control. To the residual 20 ml., 5 ml. erepsin added.

No peptide hydrolysis in 12 days; amide split 0·21 %.

Exp. 22. Erepsin-free trypsin on d-*leucyl*-l-*asparagine.* Digest: 0·6342 g. of peptide dissolved in water, neutralized and made up to 25 ml. with 5 ml. enzyme and 10 ml. veronal buffer of *p*H 8·4.

In 7 days, amide split 0·25 %, peptide split nil.

(7) *Chloroacetyl*-l-*asparagine*

Table VIII. *Exp. 23. Trypsin-free erepsin on chloroacetyl*-l-*asparagine*

Digest: 1·264 g. chloroacetyl-*l*-asparagine neutralized and made up to 25 ml. with phosphate buffer at *p*H 7·8. 10 ml. of this + 10 ml. erepsin diluted to 25 ml. formed the digest. Substrate and enzyme controls showed no change.

Time	Ammonia as ml. *N*/280 acid in 2 ml. digest	% amide split	ml. *N*/10 KOH for 2 ml. digest	% peptide split
0	0·09	—	1·43	—
6 hr.	0·15	0·22	1·65	11·3
18 hr.	0·18	0·33	2·01	29·9
24 hr.	0·24	0·55	2·04	31·5
48 hr.	0·73	2·69	2·07	33·0
96 hr.	1·40	5·15	—	—
6 days	—	—	2·92	76·8
10 days	2·76	10·20	—	—

Exp. 24. Erepsin-free trypsin on chloroacetyl-l-asparagine. Digest: 0·6515 g. substrate neutralized and made up to 25 ml. with veronal buffer *p*H 8·4. 5 ml. removed for substrate control. Residual 20 ml. + 5 ml. trypsin formed the digest.

In 7 days, amide split 0·2 %, peptide split nil.

(*8*) *Anhydroglycylasparagine*

Exp. 25. Trypsin-free erepsin on anhydroglycylasparagine. Digest: 0·7944 g. dissolved in 10 ml. phosphate buffer *p*H 7·8. 2 ml. removed for substrate control. Residue made up to 25 ml. with 10 ml. erepsin + water.

No amide or peptide split at the end of 6 days.

Exp. 26. Erepsin-free trypsin on anhydroglycylasparagine. Digest: 0·3086 g. substrate in 10 ml. water. 2 ml. removed for substrate control and the residue made up to 25 ml. with 5 ml. trypsin and 10 ml. buffer.

No amide or peptide split at the end of 7 days.

Exp. 27. Pepsin on anhydroglycylasparagine. Digest: the solution in Exp. 25 was used after inactivation of erepsin, adjustment of *p*H to 1·8 and addition of pepsin. The final strength of the solution was 0·041 *N*.

No amide or peptide split in 4 days.

Exp. 28. Papain-HCN on anhydroglycylasparagine. Digest: 0·2856 g. anhydroglycylasparagine dissolved in 10 ml. 2 ml. removed for substrate control and the residue diluted to 25 ml. after addition of 10 ml. citrate buffer *p*H 5 and 5 ml. papain solution.

No amide or peptide split at the end of 7 days.

Summary

1. The amide group in leucinamide, asparagine and glycinamide is hydrolysed by trypsin-free erepsin, the rate of hydrolysis decreasing with the three substrates in the order given.

2. Trypsin-free erepsin also hydrolyses the peptide group in chloroacetyl-asparagine, but not the amide group in either this or other asparagine peptides examined.

3. Pepsin, erepsin-free trypsin and papain are without action upon the amide or peptide groups in glycinamide, leucinamide, asparagine, glycylasparagine or leucylasparagine.

4. Anhydroglycylasparagine (2:5-diketopiperazine acetamide) is not affected by any of the animal proteases or by papain.

5. The bearing of these results on (i) the specificity of the proteolytic enzymes, and (ii) the "amide" nitrogen of proteins is briefly discussed.

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CCLXXII. PREPARATION OF SOME PROTEIN SAMPLES FROM THE FRESH LEAVES OF PLANTS AND THE SULPHUR DISTRIBUTIONS OF THE PREPARATIONS

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Both from a general standpoint and from the particular one of nutrition, the compositions of the proteins which are present in the leaves of plants are of very considerable interest. In the present article, methods for determining sulphur-distributions, particularly in their bearing upon the estimations of cystine, cysteine and methionine, are examined and applied to several leaf protein preparations.

The leaf protein preparations numbered 1, 2, 3, 5, 6, 7, 8 and 10 in Table I were made during 1935 at Adelaide, South Australia, from fresh leaf material grown on plots which had not been manured for several months. The leaves were macerated directly [cf. Osborne & Wakeman, 1920; Chibnall & Schryver, 1921; Osborne *et al.* 1921; Chibnall, 1922] in a hand-driven corn mill at room temperature (about 20°) with the solvent intended to carry the protein. Ether-water was normally used as solvent [Chibnall & Schryver, 1921] as it was thought that plasmolysis by the ether might assist the dispersion of the protoplasm. The green juice was squeezed through a cloth, filtered through layers of filter paper (which did not retain the chloroplast material very well), and the protein was flocculated with acid at the apparent isoelectric point (about *p*H 4·5) and coagulated by heating the mixture to about 90° as described by Chibnall *et al.* [1933]. The coagulum was filtered off, washed with 0·0005 *N* HCl and, further to remove impurities, extracted successively with boiling alcohol, boiling 0·005 *M* citric acid (which usually reduces the ultimate ash content but removes only a negligible amount of nitrogenous substance), twice with boiling alcohol (or until extract was almost free from chlorophyll) and finally with warm ether.

In cases 3 and 6, some of the leaf material was ground with 10% aqueous NaCl saturated with ether, instead of with ether-water as were the corresponding preparations, 2 and 5 respectively. Prep. 8 was made by extracting, but not grinding, with this solvent the leaf residues after extraction of 7. The object of using NaCl solution was to provide a satisfactory solvent for globulins in case they happened to be present. Microscopical examination of all the leaf residues revealed that large numbers of the cells had not been ruptured.

Prep. 9 had been made some years ago in this laboratory by the "used ether-water" method of Chibnall *et al.* [1933]. Prep. 4 was made by the same method from leaf material growing on a well-manured plot. The leaf material was first plasmolysed with ether-water which had already been used once for this purpose,

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the readily diffusible constituents were removed by alternate imbibition of water and expression in a Büchner press, and the residue was then macerated with water in a motor-driven mincer. After squeezing the juice through cloth and filtering through a thick pad of paper pulp the protein was flocculated at about *p*H 4·5 and coagulated by heat. Impurities were removed from the coagulum by extracting with boiling water, alcohol, acetone and ether. The filtered leaf-juice was almost devoid of green colour, suggesting removal of most of the chloroplast material. Many of the leaf cells were not ruptured by the grinding.

The proteins were all denatured in the course of preparation. As regards the proteins properly associated with the plastids and the nuclei, it is improbable that they are adequately represented in preparations obtained by either of these procedures.

In Table I are shown, in the order of the columns: the number distinguishing the preparation: the plant species and date of extraction: the % dry wt. of the leaf material: the % total N (*t*), "albuminoid" N (*a*) and coagulable N (*c*) in the

Table I

No.	Source and date		% dry wt.	% N in dry leaf (*t*, *a*, *c*)	Extraction: Protein-N as % of leaf-N (*t*, *a*, *c*)	% N protein	% ash protein
1	*Dactylis glomerata* (cocksfoot grass)	3. vi. 35	17·9	2·98 (*t*), 2·80 (*a*)	24·0 (*t*), 25·5 (*a*)	13·1	3·4
2	,,	7. xi. 35	19·0	3·20 (*t*), 2·82 (*a*)	24·4 (*t*), 27·7 (*a*)	13·0	1·0
3	,,	7. xi. 35	19·0	3·20 (*t*), 2·82 (*a*)	17·5 (*t*), 19·9 (*a*)	13·1	1·0
4	,,	29. ix. 36	12·5	6·81 (*t*), —	26·0 (*t*), —	14·1	1·1
5	*Phalaris tuberosa* (Toowoomba canary-grass)	20. viii. 35	20·4	3·08 (*t*), 2·72 (*a*)	13·4 (*t*), 15·2 (*a*)	15·4	0·7
6	,,	20. viii. 35	20·4	3·08 (*t*), 2·72 (*a*)	8·1 (*t*), 9·2 (*a*)	15·35	0·8
7	*Medicago sativa* (lucerne)	18. vii. 35	20·1	5·1 (*t*), 4·4 (*a*)	27·2 (*t*), 31·6 (*a*)	13·85	1·4
8	,,	18. vii. 35	20·1	5·1 (*t*), 4·4 (*a*)	0·95 (*t*), 1·1 (*a*)	13·85	1·7
9	,,	29. vi. 33	21·0	3·25 (*t*), 2·8 (*c*)	10·9 (*t*), 12·6 (*c*)	14·2	1·2
10	*Atriplex nummularium* (Oldman salt-bush)	6. xii. 35	15·6	3·48 (*t*), 2·54 (*a*)	13·1 (*t*), 17·9 (*a*)	12·1	4·5

dry-leaf material: the extracted protein-N as a percentage of the leaf-N (*t*, *a*, *c*): the % N in the protein preparation and finally the % of ash in the protein by ignition in air at about 600°. Materials dried in air at 85° were normally used in the analyses. Complete drying (for dry wt.) was done by heating at 105° followed by vacuum desiccation over conc. H_2SO_4. N estimations were made by the Kjeldahl method. The coagulable N was that which was not extracted from the finely-ground dried material at about *p*H 4·5 by two 10 min. extractions each with 100 times the weight of dilute hydrochloric or acetic acid at 100°, and the albuminoid N was that remaining after extraction under the Stutzer conditions ($Cu(OH)_2$ reagent).

Methods of determining the sulphur distribution

Assuming provisionally that the only S-containing amino-acids in the protein are cystine (and/or cysteine) and methionine, the distribution may be written: cystine-S + cysteine-S + methionine-S + other S = total S, and if "other S" is present entirely as SO_4 (as inorganic contaminant or attached to $-NH_2$ groups) and ethereal SO_4 (e.g. of prosthetic groups, if any), it should appear as inorganic SO_4 on prolonged acid hydrolysis at high temperature. In the absence of systematic errors it should thus be possible to check the provisional assumptions to within the limits imposed by the magnitudes of the random errors. The systematic errors, however, may be very large and they are, as yet, so uncertain

in some respects as to make a proper check of the above assumptions impossible. The sources of these errors have been classified roughly elsewhere [Lugg, 1933, 2; 1938] chiefly in relation to specific procedures such as hydrolysis, but it must be realized that sources associated with an assumed extensibility of knowledge of the behaviour of, say, a free amino-acid to the case of the corresponding amino-acid residue in the protein molecule, have an existence independent of procedure. In the following discussions they will be considered in their particular relationships to the estimations concerned.

The most specific methods of estimating cystine and cysteine present in solution appear to be the colorimetric ones. Employed in conjunction with acid (HCl, H_2SO_4) hydrolysis of "pure" proteins they have yielded valuable data, but it is known that errors may be associated with the hydrolysis of impure proteins. Bailey [1937] has shown that the losses resulting from HCl hydrolysis of edestin with arabinose are enormously greater than would be anticipated from hydrolysis tests of free cystine with arabinose and other carbohydrates [Lugg, 1933, 2], but the causes remain obscure. They may be connected with the formation, even temporarily, of sulphydryl groups by hydrolytic cleavage of disulphide bonds in edestin [Mirsky & Anson, 1934–5; Astbury *et al.* 1935; Mirsky, 1935–6], for in the presence of carbohydrates and their decomposition products the resulting mercaptal and mercaptole formation could effect large losses [Lugg, 1933, 2]. The procedure might be expected to lead to serious underestimation with the impure leaf proteins which normally contain substances of carbohydrate nature. It was found that the losses of cystine could not be averted by hydrolysing with the HCl-H.COOH reagent of Miller & du Vigneaud [1937] in place of HCl. Hydrolysis with alkali-stannite reagents could not be exploited, for whilst free cystine is almost quantitatively reduced to cysteine, edestin yields little more than half the cysteine to correspond with the cystine content of HCl hydrolysates.

Blumenthal & Clarke [1935], in a valuable contribution, estimated the following S-fractions in some protein preparations: sulphate-S after acid hydrolysis, labile S (i.e. appearing as PbS after reduction and digestion with alkali-plumbite), SO_4-S after oxidation with bromine-water, SO_4-S after oxidation with HNO_3 and non-SO_4-S after HNO_3 oxidation (i.e. by difference between total S and the preceding value). They tested the procedures, which do not permit discrimination between cystine and cysteine, with mixtures of cystine and methionine, and adjusted conditions so that the labile S and SO_4-S due to HNO_3 oxidation would correspond with the cystine present, and the non-SO_4-S after HNO_3 oxidation with the methionine, for whilst cystine-S is readily oxidized to SO_4 by HNO_3 methionine-S is only slowly oxidized beyond sulphoxide, sulphone or possibly methanesulphonic acid. Neither cystine nor methionine yield much SO_4 when treated with bromine water. As the estimated S-fractions in the proteins were not in complete conformity with the cystine and methionine contents estimated by fairly specific methods and did not show the same interrelationships as mixtures of cystine and methionine would have shown, and were often unaffected by working with HCl hydrolysates of the proteins instead of the proteins themselves, they attributed the discrepancies to the presence of small quantities of other S-containing amino-acids apart from cysteine. Such amino-acids may indeed occur in proteins, but the claim is based upon the assumption that the amino-acid residues behaved in the various procedures precisely as do the free amino-acids, and there is therefore the distinct possibility that unavoidable deficiencies of method suggested the presence of substances that did not exist in the proteins.

SO_4-S after HCl hydrolysis, SO_4-S after HNO_3 oxidation and total S estimations, which may be regarded as comprising "differential oxidation" data, were all made with the leaf proteins. The other S-fractions studied by Blumenthal & Clarke could not have been estimated satisfactorily in such preparations.

A further contribution to the study of the S-distribution in proteins was made by Baernstein [1936, 1, 2] who had earlier [1932; 1934] exploited Barger & Coyne's [1928] adaptation of the Zeisel procedure for the estimation of methionine. Baernstein found that in the course of digestion with conc. HI cystine was mainly reduced to cysteine and methionine was largely converted into methyl iodide and the thiolactone of homocysteine. Having established that the thiolactone ring would open and reduce tetrathionate to thiosulphate at mild alkalinity, he was able to devise iodine titrations for the thiolactone as well as for the cysteine. The scheme does not permit any discrimination between cystine and cysteine. He found also that SO_4 was reduced to H_2S and SO_2 by digestion with the HI. On this basis he claimed that almost all the S in a variety of proteins occurred in cystine, cysteine, methionine and SO_4, a claim since disputed by Bailey [1937]. From tests with mixtures of cystine, methionine, other amino-acids and small amounts of glucose, Baernstein believed that the procedure would be applicable without error to impure proteins.

It was found that Baernstein's [1936, 1] methods required modification before they could be applied at all satisfactorily to the impure leaf proteins, particularly in regard to the digestion with HI. His earlier (volatile iodide) method for the estimation of methionine cannot be considered in any way reliable with preparations containing polysaccharide impurity of unknown composition.

Details of procedures and tests

Hydrolysis with HCl. 2 g. samples of preps. 1 and 10 were heated with 15 ml. of 5*N* HCl at 100° for 24 hr. The mixtures were adjusted to *p*H 2·5 with NaOH, the insoluble humin was removed and the hydrolysates were adjusted to volume. The *R*SS*R* [Lugg, 1932] and cystine [Lugg, 1933, 1] contents of the hydrolysates were estimated. The *R*SS*R* estimations were performed without the aid of a compensating colorimeter to correct for the pronounced adventitious colorations, simply by making enough trials with the $HgCl_2$ and ferrous salt and then comparing the appropriate "ferrous salt standard" with a cystine standard colour solution. A compensating colorimeter was employed in the single cystine estimation. No *R*SH could be detected with certainty in the hydrolysates, but then no steps had been taken to prevent oxidation to *R*SS*R*, and in any case the impurities would have left very little *R*SH free.

Digestion with HI. Baernstein's [1936, 1] digestion procedure is to heat for 8 hr. at the boiling point in a current of nitrogen about 0·5 g. of the protein with 10 ml. of fresh, conc. HI (57 % HI, sp. gr. 1·7) from which the slight reddish coloration has first been removed by warming with 0·1 g. of KH_2PO_2. The procedure was tested with mixtures of cystine and methionine, the cystine titrations being about 2 % and the methionine about 10 % short of stoichiometrical requirements. Similar findings have recently been reported by Kassell & Brand [1938]. With recrystallized edestin the cystine plus cysteine and methionine contents, corrected for the above losses, checked fairly well with the *R*SS*R* and methionine contents obtained by Bailey [1937] for a different sample of edestin. In the presence of 20 % by weight of arabinose, however, the coloration due to iodine was not permanently discharged and there were losses of about 9 % of the cystine plus cysteine and about 8 % of the methionine; these losses were

increased to about 13 and 19% respectively (neglecting any effects of modification in the absence of arabinose) by increasing the amount of KH_2PO_2 employed from 0·1 to 0·5 g., which was more than sufficient to prevent the appearance of iodine. Complete elimination of hypophosphite during the 8 hr. digestion period proved to be entirely unsatisfactory, as also was the substitution of red phosphorus for KH_2PO_2.

The most satisfactory variation was to reduce the coloration of the HI to a deep straw-yellow by heating with the minimum amount of KH_2PO_2 necessary, and to commence the digestion of the protein with the addition of 0·02 g. of KH_2PO_2, adding like amounts as digestion proceeded and with intervals of not less than 2 min. between additions, so long as the iodine coloration continued to develop. As a rule the intervals are found to be of several hours' duration after the first few additions. It is desirable to avoid an excess of more than 0·02 g. of KH_2PO_2 at any time, and the coloration due to iodine should never be much more than noticeable. With impure leaf proteins the digest can be dark for reasons other than the presence of iodine and care must be exercised in adding KH_2PO_2. The volume of digest is most satisfactorily reduced later to about 3 ml. by evaporation under reduced pressure in a current of nitrogen with a final 0·02 g. of KH_2PO_2, and if, on subsequent dilution to 25 ml., the solution is not clear, it should be filtered in a nitrogen atmosphere. A small amount of tarry "humin" develops during the digestion of impure leaf proteins and of arabinose or pectin (especially if protein is present), and an orange coloured flocculum appears when the evaporated digest is diluted. Incidentally, more satisfactory end-points with starch as indicator are obtained in the titration of the cysteine, by adding a slight excess of the iodate solution and instead of back-titrating with the thiosulphate solution, adding a slight excess thereof and back-titrating with the iodate.

Under these modifications mixtures of cystine and methionine showed deficiencies to titration of about 2% of the cystine and about 5% of the methionine, as judged by stoichiometrical requirements. Superimposed upon the loss of 2% of the cystine by titration, some 4 or 5% of the reduced cystine (cysteine) was modified in such a way (deaminated, decarboxylated?) that it would not react with the highly specific Sullivan reagent. Baernstein [1936, 1] states that the reaction, as used by Sullivan & Hess [1930], may be applied to the cysteine in the diluted HI digests though not very satisfactorily. The reaction was found to be affected by the presence of iodides and the appropriate substances must therefore be introduced in preparing the standard used for colorimetric comparison. Plain HI-KH_2PO_2 digests and HI-KH_2PO_2 digests of gelatin plus tyrosine were used for this purpose, in conjunction with Lugg's [1933, 1] method[1] based upon the Sullivan reaction, and due allowance was made for the minute cystine content of the gelatin. The digests of gelatin plus tyrosine were used when estimating the cysteine contents of protein digests, but were not entirely satisfactory, colour-matching being imperfect.

The effects of the presence of 20% of arabinose upon the estimations of cystine plus cysteine and methionine in edestin were only slightly reduced by introducing the new digestion procedure (see Table II). The effects of 20% by weight of "Collier" pectin were about half as great as those of arabinose. There were substantial increases in the values obtained with the impure leaf proteins, however. Blanks with arabinose and pectin were virtually zero.

[1] Strangely enough it has not been recognized by some [e.g. Sullivan & Hess, 1936] that in this method equimolecular amounts of cystine and cysteine yield substantially the same coloration, in contrast to the erratic relationships obtaining in the Sullivan [1926] and Sullivan [1929] methods.

Differential oxidation

SO_4-S after HCl hydrolysis (i.e. without oxidation). 0·5 g. of the preparation was heated at 100° with 5 ml. of 5*N* HCl for 20 hr., and the mixture was adjusted to *p*H 2·5 with NaOH. After filtering, the filtrate and washings (with 0·01 *N* HCl) were treated with 0·3 ml. of 10*N* HCl and evaporated to 30 ml. on the water bath. 1·0 ml. of 0·5*M* $BaCl_2$ solution was added and the solution was kept hot for 1 hr. and then allowed to cool and to stand for 12–15 hr. The $BaSO_4$ was collected by filtration and washed free from contaminants as judged by a negative chloride test with $AgNO_3$. It was ignited and weighed in the usual way.

SO_4-S after HNO_3 oxidation. 0·3 g. of the preparation was boiled with 8 ml. of "fuming" HNO_3 (sp. gr. 1·5, 95% HNO_3) for 36 hr. in a 100 ml. round-bottomed flask fitted with a "finger" condenser, and containing a small glass bead to promote even ebullition. The mixture was evaporated with 50 mg. of KNO_3 to dryness on the water bath, and twice re-evaporated to dryness after the addition of 2 ml. of 5*N* HCl each time. It was then taken up in water containing 0·3 ml. of 10*N* HCl and, after filtering, the filtrate and washings were evaporated to 30 ml. and $BaSO_4$ was precipitated and weighed as before.

Total-S (SO_4-S after peroxide-fusion). 0·2 g. of the preparation was treated with 5 ml. of 1·5*N* NaOH in a nickel crucible and the mixture was evaporated to dryness at 105°, the drying surface being pricked occasionally to assist evaporation. It was then fused gradually under a mixture of 2 g. each of Na_2O_2 and Na_2CO_3 to a dull red heat, and a further 0·1 g. of Na_2O_2 was added before cooling. The melt was dissolved in warm water, treated with 0·1 g. of Na_2O_2 and acidified slightly by adding 10*N* HCl. After warming to expel free chlorine NaOH was added in slight excess (indicated by the persistence of precipitated nickel hydroxide) and then 10*N* HCl in 0·5 ml. excess over that required to dissolve the nickel hydroxide. The solution was filtered, filtrate and washings were evaporated to 50 ml. and $BaSO_4$ was precipitated and weighed as before.

All operations were made to conform as far as possible with standard plans, and blanks were determined by putting varying known quantities of Na_2SO_4 through the standardized operations (simple "reagent blanks" can be most misleading).[1] A few checks of the data quoted by Blumenthal & Clarke [1935] for the oxidation of cystine and methionine by HNO_3 proved satisfactory. Addition of 20% by weight of arabinose to edestin was without effect upon the values obtained with the protein itself. The presence of finely divided silica in HNO_3 digests of the impure leaf proteins may have been responsible for their occasional tendency not to boil smoothly.

Results and discussion

The protein portions proper of preparations which contain non-nitrogenous impurity are most readily compared in amino-acid composition by referring the analyses to a nitrogen basis. It is believed that none of the preparations contained more than a trace of nitrogenous impurity. The work was done with air-dried materials containing several % of moisture as a rule, but drying at 105° for 18 hr., contrary to Baernstein's [1936, 1] findings, did not affect the estimations detectably.

[1] Iron and aluminium, if present in appreciable quantity, must be removed from solution by the usual methods before precipitating the $BaSO_4$.

The results of analyses of the leaf protein preparations are shown in Table II, and are prefaced by those of edestin (A) and edestin plus 20% by weight of arabinose (B). Some of the values are from single, others from duplicate and triplicate estimations. The random errors, as % of the values reported,

Table II

		HCl hydrolysis		HI digestion			Differential oxidation			
No.	% N	% cyst.-N (*RSSR*)	% cyst.-N (sp.)	% cyst.-N (sp.)	% cyst.-N (titr.)	% meth.-N (titr.)	% cyst.-N	% meth.-N	% cyst.-N + % meth.-N	SO_4-S × 100/N
A	18·5	0·88*	0·82*	0·87	0·90	1·18	0·94	1·24	2·18	0·07
B	(15·4)	0·59†	0·59†	—	0·84	1·09	0·94	1·24	2·18	0·07
1	13·1	0·14	—	—	1·38	1·21	1·51	1·44	2·95	0·33
2	13·0	—	—	—	1·28	1·20	1·47	1·35	2·82	0·27
3	13·1	—	—	—	1·28	1·19	1·53	1·47	3·00	0·19
4	14·1	—	—	1·4	1·50	1·30	1·68	1·49	3·17	0·40
5	15·4	—	—	1·5	1·50	1·45	1·63	1·48	3·11	0·52
6	15·35	—	—	1·4	1·40	1·36	1·58	1·44	3·02	0·89
7	13·85	—	—	1·1	1·11	1·22	1·19	1·23	2·42	0·29
8	13·85	—	—	—	—	—	1·31	1·23	2·54	0·34
9	14·2	—	—	—	1·21	1·31	1·29	1·35	2·64	0·41
10	12·1	0·74	0·52	1·15	1·30	1·55	1·44	1·53	2·97	1·92

Note. Quantities marked * are recalculated, with corrections for losses of the free amino-acids, from Bailey's [1937] data for a different sample of edestin containing 18·4% N but prepared from the same batch of hemp seed by the same method, and those marked † for edestin to which 10% by weight of arabinose had been added. Bailey found 20% loss of methionine after hydrolysing the edestin-arabinose mixture with HCl and removing the humin, and no loss in absence of arabinose, the % methionine-N by Baernstein's [1934] "volatile iodide" procedure being 1·23 (recalculated with correction). Baernstein [1936, 2] reports values by titration of HI digests for two samples of edestin, the values for one of the samples agreeing fairly well with those shown above in the table.

are believed not to exceed those indicated below in the description of the contents of the table. In the order of the columns are shown: the number or letter distinguishing the preparation (see Table I): the % N in it (error 0·5%, affecting all the other errors); and then a series of estimated cystine plus cysteine and methionine contents expressed as % of the total N appearing as these amino-acids and a few quantities incidental thereto. The series is: cystine plus cysteine from *RSSR* content (error 2% for A, 5% for the others), and by specific colorimetric estimation (error 3% for A, 5% for the others) of HCl hydrolysates; cystine plus cysteine by specific colorimetric estimation (error 4 or 5% for A, 8% for the others), cystine plus cysteine from titration (error 1 or 2% for A and B, 3% for the others), and methionine from titration (error 2% for A and B, 4% for the others) of HI digests; cystine plus cysteine (error 2% for A and B, 4% for the others) and methionine (error 3% for A and B, 5% for the others) from differential oxidation on the assumption that these are the only S-containing amino-acids and that their S appropriately appears as SO_4 in the successive stages of oxidation; the sum of the last two values (error 2%); and finally the SO_4-S as % of the weight of N in the material taken (error from 2% for the larger to 20% for the smaller values). All values have been corrected only for the losses to which the free amino-acids are subject in the procedures involved, and even these corrections, though small, are not rigorously applicable.

In the first place it will be noticed that the various procedures all lead to substantially the same values in the case of edestin (A), and in the second place that the values obtained with the impure leaf proteins vary with procedure in a manner reminiscent of the effects of adding arabinose to edestin (B). The

extremely low *RSSR* content of the HCl hydrolysate of I recalls similar low cystine contents of cocksfoot leaf protein HCl hydrolysates reported by Pollard & Chibnall [1934]. From various considerations (extensive amino-acid analyses, the amounts of humin produced on acid hydrolysis etc.) it seems that the cocksfoot preparations, and probably those from other species of leaf too, should contain rather more than 16·5% N if they were "pure", as against an earlier computation by Miller [1936] of 16·0%, and that the usual organic impurity may be a pentosan, a pectin or a mucilage with the evidence mostly in favour of the last. There can be little doubt therefore that the major variations of the values with procedure, as shown in Table II, are due to the presence of organic impurities.

A greater amount of impurity may be expected to have a greater effect upon an estimation, and the degrees of vitiation may well depend upon the precise nature of the protein as well as that of the impurity. In the case of edestin itself (A), however, there are definite, small variations with procedure, and these may be attributed either to the presence of very small amounts of other S-containing amino-acids or to the deficiencies of methods of estimation which still involve the unwarrantable assumption that an amino-acid residue in protein behaves precisely as does the corresponding free amino-acid. Moreover, although the disparities between the values obtained by HI digestion and differential oxidation of the impure leaf proteins are generally greater than they are with edestin plus arabinose, it is justifiable to assume that the impurities would cause some loss in the former procedure. The differential oxidation procedure therefore, though less specific, may yield the more appropriate values both with edestin and with the impure leaf proteins. Complete acceptance of these values would imply an acceptance of the provisional assumptions as to the nature of the S fractions, recognition that the sum of the fractions thus estimated necessarily equals the estimated total S and a denial of the existence of systematic errors. At all events it seems extremely unlikely that the real cystine plus cysteine contents could have been appreciably lower than those obtained by HI digestion, because titration and colorimetric estimations check well.

Any doubt concerning the specificity of the differential oxidation procedure notwithstanding, the values obtained by it are of use in deciding whether one protein differs from another in amino-acid composition. The individual cystine plus cysteine and methionine values lack the precision which their sums possess, but these too may be used for the purpose, and it can be stated that the preparations made from one species of leaf were not all identical in composition, even when prepared from the same batch of leaf material.

The three preparations from lucerne leaves were of slightly lower cystine plus cysteine and methionine contents than the other leaf protein preparations were, but by the usual standards of animal nutritional requirements none could be adjudged deficient in its contents of these amino-acids.

Summary

The preparation of some samples of protein from the fresh leaves of cocksfoot grass, Toowoomba canary-grass, lucerne and Oldman salt-bush, is described.

Methods of determining the S-distributions of the samples are discussed and described and the results of analyses are recorded.

It is pointed out that as the cystine (and/or cysteine) contents appear to lie within the range 1·1–1·7%, and the methionine within the range 1·2–1·6% of the protein-N, none of the preparations can be regarded as deficient in these amino-acids by the usual standards of the nutritional requirements of animals.

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CCLXXIII. THE AMIDE, TYROSINE AND TRYPTOPHAN CONTENTS AND THE SULPHUR DISTRIBUTIONS (CYSTINE PLUS CYSTEINE AND METHIONINE CONTENTS), OF SOME PLANT LEAF PROTEIN PREPARATIONS

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It is the purpose of this article to record partial amino-acid and amide analyses of protein preparations made here and elsewhere from time to time from the fresh leaves of various plants at different stages of growth and growing under different climatic and manurial conditions, and to compare the preparations with one another. Some work done with a few other protein preparations is included from general interest.

The preparations

The leaf protein preparations are listed in Table I, which shows in the order of the columns: the characteristic number or letter; the species of leaf material and date of extraction; the % dry wt. of the leaf material; the % total N (*t*), "albuminoid" N (*a*), coagulable N (*c*) and protein-N (*p*) in the dry leaf material; abbreviations indicating the method of extraction; the degree of extraction, usually represented by the extracted protein-N as % of the leaf-N (*t*, *a*, *c* or *p*, preferably the last, if known), but in some cases where these data are missing, by the weight of extracted dry protein as % of the leaf dry wt. (*wt*); the % N in the protein preparation; and finally, the % of ash in it. The methods of estimating most of the quantities referred to above have been described briefly in an earlier publication [Lugg, 1938, 2]. The "protein"-N in the leaf material was that left in the residue containing the coagulable N after further extraction with alcohol, dilute citric acid, alcohol and ether, as described for the purification of most of the protein preparations mentioned in the earlier article.

Of the abbreviations, "*d*" means direct maceration of the leaves [see Lugg, 1938, 2], and "*w*1" and "*w*2" mean maceration after washing the readily diffusible solutes from the leaf cells, "1" signifying that ether, and "2" that "used" ether-water, was used to plasmolyse the cells [Chibnall, 1923; Chibnall *et al.*, 1933]. Following these abbreviations, "*w*" means that water, "*ew*" that ether-water, "*es*" that ether-saturated NaCl solution, or "*p*H 7 (or 9, 10 or 11)" that a dilute buffer solution of the indicated *p*H value,[2] was the protein solvent with which the leaves were macerated; finally "*f*" and "*c*" respectively signify

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[2] The *p*H values of the juices obtained were not estimated but were certainly lower than those of the buffers employed, juices obtained with water as solvent being of about *p*H 6. It is considered unlikely that the proteins were irreversibly dissociated.

Table I

Prep.	Source and date		% dry wt.	% N in dry leaf (*t*, *a*, *c*, *p*)	Method of extr.	Extent of extr.	% N prep.	% ash prep.
A	(Edestin)		—	—	—	—	18·5	0·2
B	(Casein)		—	—	—	—	15·05	2·0
C	(*Brassica campestris*) (Swede turnip)		—	—	—	—	13·0	16·4
1	*Dactylis glomerata* (cocksfoot grass)	3. vi. 35	17·9	2·98 (*t*), 2·80 (*a*)	*d*, *ew*, *f*	25·5 (*a*)	13·1	3·4
2	,,	7. xi. 35	19·0	3·20 (*t*), 2·82 (*a*)	*d*, *ew*, *f*	27·7 (*a*)	13·0	1·0
3	,,	7. xi. 35	19·0	3·20 (*t*), 2·82 (*a*)	*d*, *es*, *f*	19·9 (*a*)	13·1	1·0
4	,,	29. ix. 36	12·5	6·81 (*t*), 4·50 (*p*)	*w*2, *w*, *f*	39·4 (*p*)	14·1	1·1
5	,,	27. v. 37	12·4	4·15 (*t*), 3·41 (*p*)	*d*, *w*, *f*	31·1 (*p*)	11·8	4·8
6	,,	27. v. 37	12·4	4·15 (*t*), 3·41 (*p*)	*d*, *p*H 7, *f*	37·6 (*p*)	12·05	3·6
7	,,	27. v. 37	12·4	4·15 (*t*), 3·41 (*p*)	*d*, *p*H 9, *f*	37·3 (*p*)	12·7	2·7
8	,,	27. v. 37	12·4	4·15 (*t*), 3·41 (*p*)	*d*, *p*H 11, *f*	41·2 (*p*)	12·7	2·2
9	,,	20. ix. 37	16·6	5·57 (*t*), 4·33 (*p*)	*d*, *w*, *c*	30·3 (*p*)	13·1	1·0
10	,,	20. ix. 37	16·6	5·57 (*t*), 4·33 (*p*)	, *p*H 10, *c*	5·7 (*p*)	13·2	4·8
11	,,	20. ix. 37	16·6	5·57 (*t*), 4·33 (*p*)	*d*, *p*H 10, *c*	36·7 (*p*)	13·1	1·2
12	*Phalaris tuberosa* (Toowoomba canary-grass)	20. viii. 35	20·4	3·08 (*t*), 2·72 (*a*)	*d*, *ew*, *f*	15·2 (*a*)	15·4	0·7
13	,,	20. viii. 35	20·4	3·08 (*t*), 2·72 (*a*)	*d*, *es*, *f*	9·2 (*a*)	15·35	0·8
14	*Lolium perenne* (perennial rye-grass)	6. xi. 33	18·1	4·6 (*t*), 3·8 (*c*)	*w*2, *w*, *f*	21·8 (*c*)	12·85	1·1
15	*Lolium italicum* (Italian rye-grass)	12. vi. 33	16·0	— —	*w*2, *w*, *f*	4·8 (*wt*)	14·2	1·1
16	,,	26. vi. 33	15·0	5·9 (*t*), 5·2 (*c*)	*w*2, *w*, *f*	20·5 (*c*)	13·5	1·1
17	*Poa trivialis* (rough-stalked meadow-grass)	12. vi. 33	22·0	— —	*w*2, *w*, *f*	2·3 (*wt*)	12·7	1·5
18	,,	27. vi. 33	14·0	6·2 (*t*), 5·3 (*c*)	*w*2, *w*, *f*	13·7 (*c*)	14·0	0·8
19	*Festuca rubra* v. *fallax* (Chewing's fescue-grass)	26. ix. 32	—	— —	*w*2, *w*, *f*	17·8 (*c*)	14·25	0·8
20	,,	9. vi. 33	26·0	— —	*w*2, *w*, *f*	1·3 (*wt*)	14·4	2·0
21	,,	29. vi. 33	15·0	— —	*w*2, *w*, *f*	0·9 (*wt*)	14·2	1·7
22	*Cynosurus cristatus* (crested dog's tail grass)	12. vi. 33	24·0	— —	*w*2, *w*, *f*	6·6 (*wt*)	14·1	1·3
23	*Medicago sativa* (lucerne)	18. vii. 35	20·1	5·1 (*t*), 4·4 (*a*)	*d*, *ew*, *f*	31·6 (*a*)	13·85	1·4
24	,,	18. vii. 35	20·1	5·1 (*t*), 4·4 (*a*)	, *es*, *f*	1·1 (*a*)	13·85	1·7
25	,,	29. vi. 33	21·0	3·25 (*t*), 2·8 (*c*)	*w*2, *w*, *f*	12·6 (*c*)	14·2	1·2
26	*Trifolium repens* (wild white clover)	18. x. 33	13·6	5·09 (*t*), 4·3 (*c*)	*w*2, *w*, *f*	28·0 (*c*)	13·1	1·5
27	*Trifolium pratense* (red clover	23. x. 33	23·7	2·71 (*t*), 2·4 (*c*)	*w*2, *w*, *f*	24·3 (*c*)	12·75	1·6
28	*Phaseolus vulgaris* (runner bean)	7. x. 36	—	— —	*w*2, *w*, *f*	—	13·3	3·2
29	*Atriplex nummularium* (old-man salt-bush)	6. vi. 35	15·6	3·48 (*t*), 2·54 (*a*)	*d*, *ew*, *f*	17·9 (*a*)	12·1	4·5
30	*Spinacia oleracea* (spinach)	1923	—	— —	1, , *f*	*ca.* 2 (*p*)	15·5	1·3
31	,,	,,	—	— —	*w*1, *w*	—	11·7	14·8
32	,,	,,	—	— —	*w*1, *w*, *f*	*ca.* 20 (*p*)	15·1	2·1

that the leaf juice, after squeezing through cloth, was either filtered through filter paper, or centrifuged for 30 min. in a field of 500 × gravity, before flocculating the protein. Whereas most of the chloroplast and nuclear material was removed with the cell debris by filtration, much of the chloroplast material was not removed by centrifuging. In one instance, 30, protein was flocculated from the solution carrying the readily diffusible cell constituents; in another, 31, protein was flocculated from juice which had not been centrifuged or filtered through filter paper; in two other instances, 10 and 24, residues of leaf material from the extraction of 9 and 23 respectively, were extracted with other solvents but without further maceration.

The preparations were made from leaves ranging in age from very young to quite old, and in general, as regards a single species, a high % dry wt. connotes old age and relative toughness and a high % total N (ages being similar) connotes good manuring. The proteins were extracted at room temperature (10–25°). With materials of much the same degree of toughness the macerating machinery (a mincing machine or a corn mill) permitted much the same degree of maceration. Even with the tenderest leaves, however, many of the cells were not ruptured. Preps. 14–22 and 30–32 were drawn from supplies made by Prof. A. C. Chibnall and co-workers in the past. Preps. 1, 2, 3, 4, 12, 13, 23, 24, 25 and 29 have been described in an earlier article [Lugg, 1938, 2] wherein their characteristic numbers were 1–10 in succession.

Prep. A was the sample of edestin used in earlier work [Lugg, 1938, 1, 2]; B was a sample of casein used by Vickery & White [1933] and was kindly supplied by Dr H. B. Vickery; and C, a protein obtained from the juice of macerated turnips (see Williams [1917]) and believed to have a high S content, was kindly supplied by Mr F. W. Foreman.

Methods of analysis

Amide. The general scheme behind the procedure for estimating this quantity (acid hydrolysis of amide linkages and estimation of the liberated NH_3) is to insure virtually complete amide hydrolysis with minimum deamination of peptides and amino acids (notably arginine). The problem has been studied with special care by Shore *et al.* [1936], and whilst the most appropriate estimate appears to require many estimations with increasing periods of hydrolysis and an approximate elimination (by extrapolation) of the contribution of NH_3 by the deamination reactions, reasonably representative values can be obtained by a standard period hydrolysis. The procedure adopted was to heat 0·1 g. of the preparation with 5 ml. of 2*N* HCl at 100° for 3 hr. and to distill the NH_3 off at *p*H 10·0 (borate buffer), by boiling under reduced pressure at 45°, into standard acid, the excess of which was back-titrated with alkali.

The procedure was checked with edestin and edestin plus 20% by weight of arabinose, the carbohydrate being entirely without effect and therefore, presumably, introducing no new systematic errors. In comparison with values obtained by hydrolysing the leaf protein preparations for the standard (3 hr.) period, those obtained in a 2 hr. hydrolysis period were generally a few % lower, and in a 5 hr. period a few % higher; and by hydrolysing with 5*N* HCl at the boiling point for 20 hr. they were generally increased by some 20%.

Tyrosine and tryptophan. 0·2 g. of the preparation was hydrolysed with alkali stannite reagent as described by Lugg [1938, 1] and tyrosine and tryptophan were estimated in the hydrolysate by Lugg's [1937] methods. Diiodotyrosine could not be detected in a few preparations by comparing with values obtained by plain alkali hydrolysis, and was assumed to be absent in all cases. Most of the leaf protein preparations yielded small amounts of extraneous phenol during hydrolysis and the legume preparations 23, 24, 25, 26 and 28 yielded some extraneous indole as well. These substances presumably had their origin in the impurities which appear almost always to contaminate such preparations, and the legume preparations therefore must have contained some nitrogenous impurity. This non-protein-N (presumably very small in amount) has been ignored in all calculations. Whereas the phenol was readily removed by the ether extraction advocated, the indole was not adequately removed either by the ether or the toluene extraction. It slightly affected both the tint and the fading rate in the tryptophan colour reaction and so, to an unknown but

apparently small degree, vitiated the tryptophan estimations in these cases. Correction had to be made for adventitious coloration, which was more marked in the tryptophan than in the tyrosine estimations. The tyrosine and tryptophan contents of A have already been reported [Lugg, 1938, 1].

S-distribution (cystine plus cysteine and methionine). The S-distributions of A, 1, 2, 3, 4, 12, 13, 23, 24, 25 and 29 and the methods employed have been discussed in an earlier article [Lugg, 1938, 2]. Estimations were made with the other preparations by the HI digestion (titration) and differential oxidation procedures, there described.

Results and discussion

The results are recorded in Table II. The amide and amino-acid contents are all reported on a "nitrogen basis", namely, the N contained in the chemical species as % of the N in the protein preparation. This scheme facilitates comparison of the protein moieties of impure preparations if the nitrogenous impurities are negligibly small, and they are believed to have been so here. The

Table II

Prep.	% N	% amide N	% tyr.-N	% try.-N	% cyst.-N (d.o.)	% meth.-N (d.o.)	% cyst. + meth.-N (d.o.)	SO_4-S × 100/N (d.o.)	% cyst.-N (HI t.)	% meth.-N (HI t.)
A	18·5	9·63	1·80	1·09	0·94	1·24	2·18	0·07	0·90	1·18
B	15·05	—	3·14	1·17	—	—	—	—	0·18	1·77
C	13·0	—	—	—	1·36	1·65	3·01	0·35	1·24	1·62
1	13·1	5·05	2·37	1·83	1·51	1·44	2·95	0·33	1·38	1·21
2	13·0	5·11	2·32	1·82	1·47	1·35	2·82	0·27	1·28	1·20
3	13·1	5·06	2·36	1·86	1·53	1·47	3·00	0·19	1·28	1·19
4	14·1	5·26	2·34	1·82	1·68	1·49	3·17	0·40	1·50	1·30
5	11·8	5·05	2·39	1·81	—	—	—	—	—	—
6	12·05	5·35	2·40	1·86	—	—	—	—	—	—
7	12·7	5·16	2·38	1·87	—	—	—	—	—	—
8	12·7	5·38	2·38	1·86	—	—	—	—	—	—
9	13·1	4·94	2·38	1·89	—	—	—	—	1·52	1·24
10	13·2	4·48	2·09	1·65	—	—	—	—	—	—
11	13·1	5·09	2·29	1·81	—	—	—	—	1·43	1·31
12	15·4	4·85	2·48	1·86	1·63	1·48	3·11	0·52	1·50	1·45
13	15·35	5·13	2·34	1·58	1·58	1·44	3·02	0·89	1·40	1·36
14	12·85	5·02	2·26	1·74	1·65	1·60	3·25	0·37	1·26	1·16
15	14·2	4·72	2·34	1·80	1·58	1·57	3·15	0·33	1·31	1·43
16	13·5	4·70	2·33	1·74	1·70	1·69	3·39	0·58	1·42	1·50
17	12·7	5·28	2·33	1·74	1·65	1·63	3·28	0·36	1·34	1·33
18	14·0	5·13	2·31	1·80	1·52	1·66	3·18	0·33	1·36	1·50
19	14·25	4·64	2·34	1·87	—	—	—	—	1·41	1·36
20	14·4	5·12	2·36	1·88	1·54	1·58	3·12	0·79	1·31	1·41
21	14·2	4·95	2·38	1·93	1·66	1·62	3·28	0·25	1·37	1·46
22	14·1	4·73	2·36	1·96	1·65	1·55	3·20	0·26	1·50	1·41
23	13·85	5·29	2·70	<1·98	1·19	1·23	2·42	0·29	1·11	1·22
24	13·85	5·55	2·72	<1·88	1·31	1·23	2·54	0·34	—	—
25	14·2	5·16	2·74	<2·00	1·29	1·35	2·64	0·41	1·25	1·31
26	13·1	5·68	2·49	<1·88	1·23	1·33	2·56	0·30	0·99	1·23
27	12·75	5·40	2·50	1·70	1·25	1·29	2·54	0·27	1·19	1·22
28	13·3	5·08	2·36	<1·55	1·30	1·26	2·56	1·22	1·05	1·01
29	12·1	5·98	2·55	1·74	1·44	1·53	2·97	1·92	1·30	1·55
30	15·5	5·76	2·57	1·43	—	—	—	—	1·43	1·09
31	11·7	5·36	2·63	1·72	—	—	—	—	1·22	1·27
32	15·1	5·64	2·71	1·69	—	—	—	—	1·37	1·30

results have been corrected for the losses undergone by the pure, free amino-acids in the procedures concerned. The random errors of estimation are believed to lie within the limits indicated in the following description of the table's contents. In the order of the columns are shown: the characteristic number or letter;

the % N in the preparation (error 0·5 %, affecting all the others); the amide content (error 1 % for A, 1·5 % for the others); the tyrosine content (error 1 % for A and B, 2 % for the others); the tryptophan content (error 1·5 % for A and B, 3 % for the others, excepting what were almost certainly over-estimations at least several % in the case of 23, 24, 25, 26 and 28); the cystine plus cysteine content by differential oxidation (error 2 % for A, 4 % for the others); the methionine content by differential oxidation (error 3 % for A, 5 % for the others); the sum of these last two quantities (error 2 %); the SO_4-S as % of the weight of N in the preparation (error from 2 % for the larger to 20 % for the smaller values); the cystine plus cysteine content by titration of HI digests (error 1·5 % for A, 3 % for the others); and finally the methionine content by titration of HI digests (error 2 % for A and B, 4 % for the others).

The differences between the cystine plus cysteine and methionine contents estimated by titration of HI digests and by differential oxidation, have already been discussed in relation to some of the preparations [Lugg, 1938, 2]. The disparities are again apparent with the rest of the preparations and the same uncertainty as to their significance remains. Again it may be pointed out that while the estimations by titration of HI digests are much the more specific they are likely to be low, and that the values obtained by differential oxidation must be used when comparing compositions. SO_4-S values are regarded here simply as measures of some impurity.

Whereas significant differences in composition, even in respect of only one chemical species, prove the non-identity of the protein moieties of two preparations, their absence leaves only an indeterminate degree of probability (increasing with the number of chemical species considered) that they are identical.

Of the preparations from cocksfoot grass, 5, 6, 7 and 8 show that slightly increased extraction may result when the *p*H of the extracting liquid is increased beyond that of the mixed cell contents. 5 is of slightly lower amide content. Incidentally, when leaves of the Gramineae are macerated alone or with water the resulting juice is usually found to be of about *p*H 6. Prep. 10, made by extracting the leaf residue from 9 with a *p*H 10 buffer, is of materially lower amide, tyrosine and tryptophan contents than any of the other cocksfoot preparations which, except for small differences, are remarkably uniform in composition. In fact, except for 10, all the preparations from leaves of the Gramineae (1–22) show relatively small variations in composition, the greatest being in the S-distributions and the amide contents.

In comparison with these, the preparations from leaves of the Leguminosae (23–28) are rather less uniform in composition, but in general, their tyrosine contents are a little higher and their cystine plus cysteine and methionine contents are lower; and the preparations from leaves of the Chenopodiaceae (29–32) are generally of higher amide and tyrosine contents.

No definite variations in composition of extracted protein can be associated with the age of the leaves or with the climatic and manurial conditions during growth. It is to be noted too, that preps. 9, 11 and 30, which presumably contained more representative amounts of the proteins associated with the chloroplasts, do not differ markedly in composition from the others.

Taken as a group for comparison with preparations like A and B, the leaf protein preparations from monocotyledons (1–22) and dicotyledons (23–32) appear from these analyses to be of fairly uniform composition, and judged by the usual standards of animal nutritional requirements are by no means deficient in their contents of tyrosine, tryptophan, cystine plus cysteine and methionine.

The analyses of A and B are in reasonable agreement with the more recently published work of others on edestin and casein preparations. There appears to be nothing remarkable about the S distribution of C (from Swede turnips), but then, contrary to expectation, its S content was not abnormally high.

Summary

Estimated amide, tyrosine, tryptophan, cystine (and/or cysteine) and methionine contents of protein preparations made from the fresh leaves of various mono- and di-cotyledonous plants, are recorded, together with those of a few other protein preparations.

The leaf protein preparations were made from *Dactylis glomerata*, *Phalaris tuberosa*, *Lolium perenne*, *L. italicum*, *Poa trivialis*, *Festuca rubra* var. *fallax* (Hack) and *Cynosurus cristatus*, of the Gramineae; *Medicago sativa*, *Trifolium repens*, *T. pratense* and *Phaseolus vulgaris*, of the Leguminosae; and *Atriplex nummularium* and *Spinacia oleracea*, of the Chenopodiaceae. For the most part they represented some 10–40% of the leaf protein, but were probably rather deficient in the proteins associated with the plastids and nuclei of the leaf cells.

In comparison with preparations from the first plant order, those from the second and third were generally a little higher in tyrosine contents, those from the second a little lower in cystine (and/or cysteine) and methionine contents, and those from the third a little higher in amide contents. The ranges over all the leaf protein preparations were: 4·70–5·98% amide-N, 2·09–2·74% tyrosine-N, 1·43–1·98% tryptophan-N, 0·99–1·70% cystine (plus cysteine)-N, and 1·01–1·69% methionine-N, the lower limits in the last two cases probably being underestimations, and the upper limits being possibly too high owing to lack of specificity in estimation in these same cases.

In regard to their contents of the amino-acids here estimated, the leaf protein preparations compare favourably with many other proteins which enter into the dietary of animals.

It is a pleasure to record my indebtedness to Prof. A. C. Chibnall for his continued interest and co-operation, to Mr G. A. Stroud for valuable assistance, and to the Agricultural Research Council for a grant.

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CCLXXIV. ON CERTAIN SIMPLE PEPTIDES OCCURRING IN MARINE ALGAE

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Some time ago Haas & Hill [1931; 1933, 1] described the isolation of water-soluble peptides from the brown alga *Pelvetia canaliculata* forma *libera* S. M. Baker and the red alga *Corallina officinalis* Linn. It was then suggested that the presence of peptides in algae was due to a lack of metabolic balance determined either by desiccation or by low illumination. To obtain further evidence in support of this it was decided to examine as many algae as possible and preferably calcareous forms, as these, owing to their incrustation, would naturally be exposed to a much reduced illumination. Accordingly the following were selected: *Corallina squamata* Ellis, *Lithophyllum incrustans* Foslie, *Amphiora capensis* Aresch and *Galaxaura subverticillata* Kjell. A number of unencrusted algae were also examined, but, so far, the only two found to contain peptides were *Pelvetia canaliculata* forma *libera* and *Griffithsia flosculosa*.

The peptides contained in the above calcareous algae and that of *Pelvetia* are described in the present communication with special reference to the number and nature of their constituent amino-acids, but an account of the observations made on *Griffithsia flosculosa* will be the subject of a future paper.

Methods

The general procedure for the isolation of the peptides was as follows. The air-dried material was digested three times in water over a boiling water bath for about 30 min. The resulting extract was poured off and the residual plant material freed from adherent liquid in a tincture press. The combined extracts were next precipitated with basic lead acetate, and the filtrate, after freeing from Pb with H_2SO_4, was treated with $Ba(OH)_2$ to remove SO_4 and was concentrated under reduced pressure; the solution was then precipitated by mercuric acetate, and the Hg salt, after washing, decomposed with H_2S, filtered from HgS and evaporated to dryness in a vacuum.

Nature and properties of the peptides

The peptides prepared as above are all amorphous, more or less viscous substances, varying in colour from a light lemon-yellow to a dark brown. They all give a green colour with Bial's reagent for pentose, but the intensity of the reaction varies, being least for *Pelvetia* and highest for *Lithophyllum*. Although detectable by means of the above colour reaction, the actual amount of carbohydrate was too small to estimate in the material available.

The peptides all give the biuret reaction, the colour varying from a reddish mauve to a blue-violet colour, but as the tint varies somewhat with dilution the reaction is of no value for characterization.

With the object of obtaining some information on the number of amino-acid molecules forming the peptide chain, the ratio of amino-N before and after

hydrolysis was determined. In order to eliminate the disturbing influence of NH_3 in the determination of amino-N, the following procedure was adopted.

A given amount of peptide was made up to a known volume, and two equal aliquots were measured out. One was then placed in a flask with dilute aqueous Na_2CO_3 and heated at 60° while a rapid current of air was drawn through. After all ammonia had been removed, the contents of the flask were carefully washed out, neutralized with acetic acid, evaporated and finally washed into a graduated flask and made up to 25 ml. The amount of amino-N in this solution was then determined by the Van Slyke method.

The other aliquot was heated with twice its volume of conc. HCl in an autoclave at 105° for 4 hr. After evaporating the HCl the residue was dissolved in water, made alkaline with Na_2CO_3 and heated over a water bath until no more NH_3 was evolved; it was then acidified with acetic acid, made up to 25 ml. and the amino-N determined.

From these two determinations the number of amino-acid groups in the peptide chain was obtained.

Products of hydrolysis of the peptides

The hydrolyses were carried out as described above and the dark brown solutions obtained were evaporated to dryness over a water bath to remove excess of acid, taken up with water and filtered from humic matter. As the quantity of material available was relatively small, the ordinary large scale methods for isolating and identifying the various acids were impracticable, and for this reason the tests employed are given in detail.

Pelvetia canaliculata forma libera. This alga was collected at Blakeney Point, Norfolk, during the month of April.

The crude peptide obtained was a light lemon-yellow syrup; the yield, calculated on the air-dried alga, was 0·728 %.

After hydrolysis and removal of excess acid by evaporation the solution was tested for phenylalanine (Kapeller-Adler test) and tyrosine (Mörner's test), in both cases with negative result.

After removal of ammonia, a portion of the resulting solution was tested with phosphotungstic acid and, as no precipitate resulted, the remaining solution was concentrated to small bulk and saturated with HCl to remove glutamic acid, whose presence among the products of hydrolysis had already been established on an earlier occasion [1931].

In order to determine whether any other amino-acid remained after complete removal of the glutamic acid, the filtrate was concentrated, again saturated with HCl and once more filtered from a small quantity of precipitated glutamic acid hydrochloride; it was then boiled to drive off the acid, diluted and heated with excess of ZnO. After 40 hr. the solution was filtered and found still to give a strong ninhydrin reaction. A control experiment on authentic glutamic acid treated in the same way gave no ninhydrin reaction, showing that glutamic acid can be completely removed by this method. It was therefore concluded that a second amino-acid must be present. The solution was accordingly concentrated, freed from NH_3 and treated with a few crystals of phenol and an excess of NaOCl; an immediate deep blue colour resulted, suggesting glycine, alanine or leucine. To distinguish between these a little of the concentrated solution was treated with a few drops of saturated copper acetate; as no precipitate resulted, glycine and leucine were excluded, and it only remained to establish the presence of alanine. To this end the solution was deaminated with HNO_2 and tested for the presence of lactic acid, after treating with conc. H_2SO_4, by Hopkins' test with

copper sulphate and thiophene. A positive result was obtained, thereby establishing the presence of alanine.[1]

The ratio of amino-N before and after hydrolysis was found to be 11. In the earlier communication [1931] this ratio was given as 8 and the only acid detected was glutamic acid. Whether this discrepancy is due to seasonal variation or not is now under investigation.

The five following algae are all encrusted forms, but, as will be seen from the results described below, there is considerable variation in the composition of their constituent peptides.

Corallina squamata. The material used for extraction of the peptide was a composite sample, collected at different times of the year, from Dancing Ledge, Dorset.

The peptide was a dark amber-coloured substance of a resinous consistency, which however readily melted, on warming, to a viscous liquid. The yield of crude peptide calculated on the dry weight of the alga was 0·1 %.

After hydrolysis, the solution was treated with phosphotungstic acid; the heavy precipitate produced was washed and suspended in acetone-water and treated with excess of baryta solution; a rapid current of air was drawn through until all NH_3 had been removed; excess of baryta was removed with CO_2, and the resulting solution was shown to contain arginine by both the Sakaguchi test and Harden's diacetyl test.

The filtrate from the phosphotungstic acid precipitate was then treated with baryta to remove excess phosphotungstic acid and the precipitate, consisting of Ba phosphotungstate and $BaSO_4$, was filtered off. The filtrate gave only a feeble ninhydrin reaction, which suggested adsorption on the precipitate; the latter was therefore boiled with a strong solution of Na_2CO_3, and filtered; the filtrate now gave a strong positive reaction. After neutralization it was tested for aspartic and glutamic acids, but with negative results. On the other hand it gave a blue colour with phenol, followed by NaOCl, indicating glycine, leucine or alanine. Since it gave no precipitate with copper acetate the two former acids were excluded. The presence of alanine was, however, established by deamination with HNO_2, followed by Hopkins' thiophene test for lactic acid.

The test for phenylalanine (Kapeller-Adler) was negative.

The failure to find aspartic acid, after repeated trials, among the products of hydrolysis of this alga is significant in view of the fact that aspartic acid was definitely established in the other species, namely *Corallina officinalis*, as reported on an earlier occasion [Haas & Hill, 1933, 2].

The ratio of amino-N before and after hydrolysis of the peptide of *C. officinalis* was 4.

Lythophyllum incrustans. The material used for this investigation was collected at Studland Bay, Dorset, in May, by chipping off the mauve superficial layer covering the chalk which is exposed only at low spring tide.

The thickness of the actual algal covering was only a few mm. and the main bulk of the sample was composed of the rock substratum. The material was crushed into small fragments and extracted three times with hot water, the aqueous extract being worked up in the usual manner. The weight of crude peptide obtained from different samples varied considerably from 0·055 to 0·185 %. These figures are only approximate and depend entirely upon the amount of chalk or limestone adhering to the weed.

[1] A separate experiment showed that authentic phenylalanine when treated as above likewise gives a positive reaction. Since however the solution obtained from the hydrolysed peptide failed to give the Kapeller-Adler reaction for phenylalanine it was concluded that the presence of alanine was established.

The crude peptide was a dark brown substance of a gummy consistency. After hydrolysis it gave a precipitate with phosphotungstic acid, which after the usual treatment yielded a residue which gave good positive tests for arginine by the Sakaguchi test and by Harden's test with diacetyl. The filtrate after the usual treatment for the removal of Ba gave a positive reaction for phenylalanine (Kapeller-Adler).

The ratio of amino-N before and after hydrolysis was 4.

Amphiora capensis. This alga is a South African species allied to *Corallina*.

The air-dried material, extracted in the usual manner, yielded just under 0·3 % of crude peptide, in the form of a dark brown semi-solid residue with a peculiarly unpleasant smell.

The amount of material available was, unfortunately, not sufficient to allow of a detailed examination, and the only amino-acid which could be definitely established was arginine. The filtrate remaining after removal of the arginine gave a blue colour with phenol and NaOCl, suggesting glycine, alanine or leucine. The addition of saturated copper acetate to the concentrated solution gave a turbidity on standing, much as leucine does, but it was not found possible to establish the presence of this amino-acid conclusively. Lack of material rendered it impossible definitely to exclude glycine or alanine, or to make a determination of the ratio of amino groups before and after hydrolysis.

Galaxaura subverticillata. This is an encrusted green alga from Florida.

Extraction by the usual method gave a yield of about 0·2 % of a dark brown semi-solid peptide. The amount of material available was small, and only one amino-acid, namely phenylalanine, was definitely established. The hydrolysed product was precipitated with phosphotungstic acid without previous removal of ammonia; the resulting precipitate gave a negative test with ninhydrin, and as it gave no positive test for proline it was rejected.

The filtrate from the phosphotungstic acid precipitate was treated with baryta to remove excess of phosphotungstic acid and filtered. The filtrate was freed from Ba and precipitated with mercuric acetate. The resulting precipitate was decomposed with H_2S and filtered. This filtrate gave an amorphous precipitate with copper acetate, which was not further characterized owing to lack of material. The filtrate from the mercuric acetate precipitate, however, gave positive tests for phenylalanine. It may therefore be concluded that *Galaxaura* peptide contains at least two amino-acids of which one is phenylalanine.

The ratio of amino-N before and after hydrolysis was 2.

In addition, a further calcareous alga of South African origin, namely *Cheilosporium corymbosum*, was examined, but the amount of material available was too small for detailed examination. All that was established was that the weed yielded about 0·1 % of crude peptide in the form of a dark brown resinous material in which the ratio of amino groups before and after hydrolysis was 2.

For convenience of reference the results obtained are summarized in the following table:

	Alanine	Arginine	Aspartic acid	Glutamic acid	Phenyl-alanine
Amphiora capensis	+	+	.	.	.
Corallina squamata	+	+	.	.	.
Corallina officinalis	.	.	+	.	.
Galaxaura subverticillata	.	.	.	.	+
Lithophyllum incrustans	+	+	.	.	.
Pelvetia canaliculata forma *libera*	+	.	.	+	.

Although various other amino-acids were tested for, only those which gave positive results are recorded in the above table.

SUMMARY

1. The occurrence of peptides is reported in marine algae: *Pelvetia canaliculata* forma *libera* (Phaeophyceae), *Corallina officinalis*, *Corallina squamata*, *Amphiora capensis*, *Cheilosporum corymbosum*, the two latter from South Africa, *Lithophyllum incrustans* and *Galaxaura subverticillata* from Florida. Of these algae all except the first are encrusted forms.

2. The amount of crude peptide occurring in the algae varies from 0·05 to 0·29 % of the dry weight; in *Pelvetia*, however, the amount is as high as 0·728 %.

3. The peptides are all water-soluble and diffusible substances; they all contain a small proportion of pentose sugar.

4. The following amino-acids have been shown to occur in these compounds: alanine, arginine, aspartic acid, glutamic acid and phenylalanine.

5. In one genus, namely *Corallina*, a difference in composition of the peptide has been noted in different species, *C. officinalis* containing aspartic acid, while *C. squamata* does not, but contains instead alanine.

6. The number of peptide linkages as expressed by the ratio of amino groups before and after hydrolysis varies in the different peptides.

In conclusion we take this opportunity of acknowledging our indebtedness to Prof. T. A. Stephenson and Dr Janet Maclagan for supplying the South African algae, and to Dr F. C. Steward for the specimen of *Galaxaura* from Florida.

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CCLXXV. VITAMIN E DEFICIENCY IN THE RAT

III. FERTILITY IN THE FEMALE

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THE consumption of a diet free from vitamin E produces sterility both in the male and in the female rat. In the male the sperms become non-motile and this is followed by degeneration of the testes. The condition in the male is not reversible and cannot be cured by administration of vitamin E once the degenerative phase has been reached [Evans, 1925; Mason, 1926; Evans & Burr, 1927]. In the female it has generally been accepted that ovulation, fertilization and implantation remain normal, but pregnancy cannot be carried to term unless the animal is given an adequate dose of vitamin E on mating.

Bacharach *et al.* [1937] and Bacharach & Allchorne [1938] observed that the incidence of fertile matings among vitamin E-deficient rats was very much lower in those which had undergone a resorption-gestation than in virgin animals; they concluded that the process of resorption left some permanent damage to the reproductive system but made no histological examination. Martin & Moore [1936] reported the brown appearance of the uterus of rats fed on a vitamin E-deficient diet. Evans [1928] found that the incidence of spontaneous deciduomata of the uterus was much higher in vitamin E-deficient than in normal females.

In this laboratory it has been found that when vitamin E-deficient rats are mated with normal males and a vaginal plug is formed, this mating is almost invariably followed by implantation. I have found no apparent difference between virgin and resorbed animals in this respect. I do, however, find that among vitamin E-free animals, whether virgin or resorbed, more than half do not mate or else no plug is formed. It has also been found, in agreement with Bacharach, that animals which have once resorbed require a larger dose of vitamin E to prevent resorption in the next gestation than their litter-mate virgin sisters of the same age.

A post-mortem examination has been made of rats which have been used for vitamin E experimental work and it has been found, without exception, that every rat which has undergone a resorption-gestation has a brown or yellow-brown uterus and ovaries containing large corpora lutea. Histological examination of the uterus reveals occasional patches of fatty degeneration in both the longitudinal and circular muscle coats. Deposits of red-brown pigment are found throughout the muscle layers.[1] Many fibres are degenerated and packed with granules of pigment. These findings account for the discoloration of the uterus. There is often some fibrous tissue in the muscle layers, particularly in the older rats.

[1] Since the completion of this paper Martin & Moore have described the finding of granules of pigment in the uterine muscle of their vitamin E-deficient rats (*Chem. & Ind.* 15 Oct. 1938).

A number of virgin animals which had been fed on the vitamin E-free diet for different periods of time have been killed and examined *post mortem*. From these examinations it has been found that similar changes take place in virgin, vitamin E-free rats, but that resorption greatly accelerates their onset. Table I

Table I

No. of rats	Months on vitamin E-free diet	History	Appearance of uterus	Microscopic examination of uterus
5	4	1 resorption	Pale yellow; 1 animal had small fibroid	Some pigment in muscle layers
4	4	Virgin	Thread-like, very pale yellow; 1 brownish-yellow and infected	Traces of pigment
5	5	1 resorption	Yellow-brown; 1 showed some thickening	Considerable amount of pigment
3	5	Virgin	Yellow-brown; 2 had fibroids and were bleeding	Considerable amount of pigment
7	7	1 or more resorptions	Yellow-brown to dark orange-brown, 1 infected	Large amount of pigment
6	7	Virgin	Yellow-brown	Less pigment than resorbed animals, no fibrosis
9	8	1 or more resorptions	Brown to dark brown; 1 bleeding from fibroid, 2 infected, 3 fibrosis; all enlarged	Large amount of pigment, extensive muscle degeneration
5	8	Virgin	Pale orange-brown; 1 very distended	Pigmented, a number of pigmented and degenerated muscle fibres present; some fat and in one case mucosa oedematous

illustrates these points; it is composed from the records of 44 consecutive post-mortem examinations. After 4 months on the diet there is a slight increase in the size of the uterus and definite, although slight, discoloration; after 5 months the discoloration and enlargement are more marked and the condition is severe after 6 months of vitamin E deficiency. The uterus of a rat which has been on the diet for 5 months and undergone a resorption-gestation shows more discoloration than that of a similar virgin animal. The amount of discoloration present was in each case comparable with the amount of pigment found on microscopic examination. The enlarged condition is not due to infection, although this may sometimes occur. Where there is no infection the enlargement is usually associated with fibrosis and thickening of the uterine wall. The infections which quite frequently occur are possibly due to the frequent vaginal examinations which are made, although every reasonable precaution is taken to avoid infecting the animal.

In view of the changes in the uterine muscle it is not surprising that vitamin E-deficient animals which have undergone a resorption-gestation require more vitamin E to enable them to produce a litter than similar animals which have not resorbed, and it can also be understood that if the uterine degeneration has proceeded far enough, pregnancy will be impossible. Table II illustrates these points. It will be seen that 0·35 ml. of a (diluted) vitamin E preparation was an inadequate dose for a virgin animal which had been on the vitamin E-free diet for 9 months, but 0·5 ml. produced a litter of 6 in a similar animal. 0·5 ml. was inadequate for animals of almost the same age which had undergone one or two resorptions respectively, yet 0·75 ml. produced a litter of 1 in an animal which

Table II

Rat no.	Dose of vitamin E preparation ml.	Result	No. months on vitamin E-free diet	No. resorption gestations	Appearance of uterus
1272	0·35	Litter of 5	5	1	Slightly yellow
1136	0·35	Litter of 2	8	0	Not killed
1096	0·35	Litter of 8 (dead in uterus)	9	0	8 full-term dead foetuses removed
1022	0·50	Litter of 6	9	0	Small and brown
794	0·50	Resorption	10	1	Brown; signs of resorption of large foetus
1033	0·50	Resorption	10	2	Large and very brown
698	0·50	Resorption	11	1	Large and very brown
1051	0·75	Litter of 1	10	2	Normal colour, very small
1032	0·75	Resorption	10	2	Dark orange-brown

had had two resorption-gestations and been on the diet for 10 months, but failed to prevent resorption in an exactly similar animal. Since the 0·35 ml. dose was adequate for the 3- and 4-months animals then 0·75 ml. is a relatively enormous dose and the fact that it failed to produce a litter in an animal which had received the vitamin E-deficient diet for 10 months and gave only a litter of one in another animal which had been on the diet for the same length of time, shows that after about 10 months of vitamin E deficiency female rats have reached or are very near the stage of irreversible sterility. On a normal diet a female rat is capable of producing living young until she is at least 18 months old. The normal colour of the uterus of the rat which had a litter of 1 following a dose of 0·75 ml. remains unexplained unless the large amount of the vitamin given exerted a curative effect on the degeneration. Investigation of this point is at present in progress.

The fact that fibrosis of the uterus often occurs in connexion with the discoloration has already been mentioned, but more advanced stages than this are regularly met with in this stock of vitamin E-deficient rats. Non-pregnant animals have occasionally been found bleeding from the vagina, the amount of blood lost being quite large in some cases. 30 cases of such bleeding have been observed in the last year and reference to Table I will give some idea of its incidence. These animals have been killed and the uterus has been found to have a series of rounded whitish enlargements separated by discoloured muscle. These masses are hard to cut and their inner surfaces are pearly white. On microscopic examination they are seen to consist of a mass of fibrous tissue and are typical fibromyomata.

It is, of course, quite possible that fibromyomata occur in rats fed on a normal diet, but the incidence among our stock must be very much less than among the vitamin E-deficient rats in this laboratory, because a post-mortem examination is made of all the stock animals which die or which are of no further use for breeding, and no tumour of this type has been found on microscopic examination. Similarly, these stock animals never develop uterine discoloration and even among those animals which are too old to bear litters no pigmentation or fatty degeneration of the uterus has been found. These observations are based on the records of our breeding stock for the last 2 years. This stock has been maintained at a level of approximately 150 females, 100 of which have been killed in the interval.

These observations raise several points. It is clearly important in assaying a substance for its vitamin E activity to make sure that the animals used have been

fed on the vitamin E-deficient diet long enough to be sterile but not long enough to have developed uterine changes, and the onset of these degenerative changes must obviously vary between different stocks and depend to a great extent on the degree to which the diet is free from vitamin E.

The statement that vitamin E deficiency is reversible in the female but not in the male is only partly true, because the deficiency is curable in both male and female rats if vitamin E is given early enough, but if the deficiency is allowed to proceed to testicular or advanced uterine degeneration, absolute sterility is produced in both sexes.

A more detailed study is being made of the incidence of the fibromyomata but this preliminary work indicates that in the rat they are associated with vitamin E deficiency.

SUMMARY

The discoloration of the uterus described by Martin & Moore in vitamin E-deficient rats has been confirmed.

This discoloration is caused by the deposition of a pigment in the muscle layers associated with degeneration of the muscle.

Prolonged vitamin E deficiency caused fibrosis of the uterine muscle; a certain number of the animals developed fibromyomata of the uterus.

I wish to express my thanks to Dr S. W. F. Underhill and Dr F. H. Carr for their helpful advice and to the Directors of The British Drug Houses Ltd. for permission to publish this work.

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CCLXXVI. THE CHLORINE-SODIUM SULPHITE COLOUR REACTION OF WOODY TISSUES

II. THE BEARING OF THE COLOUR REACTION ON THE CONSTITUTION OF HARDWOOD LIGNIN

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(*Received 8 October 1938*)

In a previous communication from this Laboratory [Campbell *et al.* 1937] it was shown that the well known colour reaction given by woody tissues with sodium sulphite following chlorination was not characteristic of lignin only but could also be given by certain tannins. It was concluded that the reaction was probably specific for phenolic compounds containing the 1:2:3-trihydroxybenzene nucleus but, although such a nucleus is known to occur in the gallotannins, there is no evidence that it occurs as such in native lignin. Great interest is therefore attached to the isolation by Freudenberg [1938] of substances containing a modified 1:2:3-trihydroxybenzene nucleus such as syringic acid and also to the identification by Leger & Hibbert [1938] of pyrogallol 1:3-dimethyl ether among the degradation products of lignin. It was considered that, if any of these substances could be shown to give the characteristic colour reaction with chlorine and sodium sulphite, additional proof would be forthcoming for the occurrence in native lignin of a modified trihydroxybenzene nucleus as a recurring unit for the reason that, during the delignification of hardwoods by the Cross and Bevan procedure, the coloration persists until the last traces of lignin have been removed. This would, of course, involve the assumption that lignin is composed for the most part of aromatic units. Until the publication of the results of Harris *et al.* [1938] such an assumption could not be maintained with any certainty. In their study of the products of the hydrogenation and hydrogenolysis of hardwood lignin these authors have confirmed and extended the work of Moldavskiĭ and Vaĭnshteĭn [1935] and proved beyond doubt that the greater part of lignin is aromatic in character. In the present study chlorine and sodium sulphite have been applied to a further range of substances, particular attention being paid to products obtained by Freudenberg [1938] in view of their importance as likely building units in lignin.

Experimental

The following substances were exposed to gaseous chlorine followed by sodium sulphite in the manner previously described [Campbell *et al.* 1937] with the results shown in Table I.

The faint reaction of pure 1:2:3-trimethoxybenzene is probably associated with the low solubility of this compound in water and the consequent difficulty in securing its optimal reaction with chlorine. It was observed that of all the substances so far tested gallic, trimethylgallic and syringic acids gave colorations

Table I. *Colour reactions of various phenols and their derivatives with chlorine and sodium sulphite*

Substance	Colour reaction
Guaiacol	Nil
Vanillin	Reddish brown
Veratrole	Nil
Eugenol	Very faintly positive
Gallic acid	Strongly positive
1:2:3-Trimethoxybenzene	Faintly positive
Trimethylgallic acid	Strongly positive
Syringic acid	Strongly positive

which were most reminiscent of the deep magenta colour given by hardwoods and isolated hardwood lignin.

In certain cases it was found that a magenta coloration could be produced by reagents other than chlorine and sodium sulphite. For instance it was given by gallic and syringic acids on adding a trace of $FeCl_3$ in the presence of either Na_2SO_3 or $NaHCO_3$. Gallic acid solutions also give the coloration (1) when aerated in the presence of $NaHCO_3$ and Na_2SO_3, (2) with a trace of K_2CrO_4 and Na_2SO_3, (3) with a trace of $CuSO_4$ and Na_2SO_3.

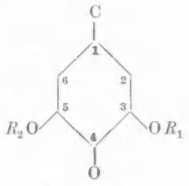
In contradistinction to gallic acid, in which the three OH groups are free, and to syringic acid, which has one free OH group, it was found that trimethylgallic acid, which has no free OH groups, could only give the colour reaction with Na_2SO_3 after previous chlorination. The same has been found to be true of hardwood lignin and, in view of the possible significance of this analogy, the behaviour of pure trimethylgallic acid on chlorination was investigated in detail as follows.

10 g. of the acid were dispersed in 15 ml. of warm water and exposed to a slow stream of chlorine for 30 min. The mixture was cooled and the solid matter (11 g.) was separated by filtration and extracted with light petroleum (B.P. 40–60°). The crystalline substance which settled out from the petrol and was recrystallized from CCl_4 consisted of colourless needles (M.P. 120°) and was soluble in EtOH, Me_2CO, $CHCl_3$, C_6H_6 and CCl_4. The substance dissolved in $NaHCO_3$ with the evolution of CO_2 and its equiv. wt. by titration was 270. ($C_6Cl_2(OCH_3)_3.COOH$ requires equiv. wt. 281; $C_6HCl(OCH_3)_3.COOH$ requires equiv. wt. 247·5.) No coloration was given by either $FeCl_3$ or $FeFe(CN)_6$, indicating that the substance contained no phenolic OH groups. It therefore probably consisted of a mixture of mono- and di-chlorotrimethylgallic acids. Contrary to expectation this chlorinated compound, which represented by far the greater part of the original trimethylgallic acid, gave no coloration with sodium sulphite. It was found, however, that the clear aqueous filtrate obtained after the original chlorination did give the coloration. A test for phenols in this filtrate gave a positive result. Repeated chlorination of the already chlorinated trimethylgallic acid gave rise to further small amounts of a phenol which gave an intense magenta coloration with sodium sulphite.

Discussion

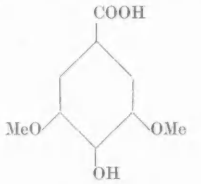
It is now realized that the chlorine : sodium sulphite colour reaction is not specific for compounds containing the 1:2:3-trihydroxybenzene nucleus itself since it can also be given by substances in which this nucleus has been modified by substitution. It is worthy of note, however, that so long as at least one OH group remains unsubstituted the colour reaction can be produced by oxidizing agents other than chlorine and weak alkalis other than sodium sulphite. When,

on the other hand, all three OH groups are alkylated chlorination is a necessary antecedent to the production of the magenta coloration with sodium sulphite. In this latter case the major reaction product is a chlorinated derivative of the starting material, but the colour reaction is given by traces of a phenolic compound which is also produced during chlorination. In the case of trimethylgallic acid it must be concluded that chlorine has acted as a demethylating, as well as an oxidizing agent. So far as is known chlorination must always be applied to hardwood lignin before the characteristic coloration is given in the presence of sodium sulphite. By analogy it is therefore strongly suggested that lignin contains a modified pyrogallol nucleus as a recurring unit. This unit may be given the general formula (I).

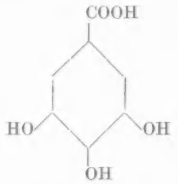


Formula (I)

A magenta colour reaction in the presence of sodium sulphite could be given by such a unit if it were transformed into syringic acid (formula II) or gallic acid (formula III).



Formula II
syringic acid



Formula III
gallic acid

In the light of the degradation studies of Freudenberg [1938], Leger & Hibbert [1938] and suggestions in the literature by Klason [1930], Wacek [1930] and others, it is reasonable to assume that R_1 and R_2 in formula (I) are OCH_3 groups and that side chains occur at positions 1 and 4. These side chains could form linkages between essentially similar methylated pyrogallol nuclei.

Summary

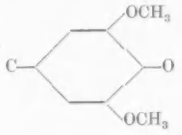
1. Continuation of previous work [Campbell *et al.* 1937] indicates that the chlorine : sodium sulphite colour reaction of hardwoods is not specific for compounds containing the 1:2:3-trihydroxybenzene nucleus itself, since it can also be given by substances in which this nucleus is modified by certain substituents.

2. Provided that at least one OH group of a pyrogallol derivative is free the colour reaction is given by oxidizing agents other than chlorine in conjunction with weak alkalis other than Na_2SO_3.

3. If all three OH groups are methylated chlorination is a necessary antecedent to the production of the coloration in the presence of sodium sulphite.

4. The major product of the chlorination of trimethylgallic acid is a crystalline chlorinated derivative of the starting material which gives no coloration with Na_2SO_3. The coloration is given by a secondary product of chlorination which is shown to be a phenol.

5. The analogy with respect to this colour reaction between certain gallic acid derivatives and hardwood lignin is discussed, and it is suggested that hardwood lignin contains a recurring unit of formula



The authors are indebted to the Director of Forest Products Research for permission to communicate these results.

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CCLXXVII. THE BODY FATS OF SOME SEA-BIRDS

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(*Received 11 October 1938*)

In earlier papers [e.g. Lovern, 1937] the writer has discussed specific features in fats which are largely independent of the diet of the animal, and Hilditch & Lovern [1936] have considered the relationship between species and fat composition. The broad schemes suggested in this second paper are not, however, free from exceptions, e.g. the whales of the Balaenidae family have fats closely resembling those of fish to which they are not, of course, zoologically related. Without, for the present, considering the matter in detail, it is evident that the diet of marine organisms is ultimately responsible for the fat of these whales having such a composition.

Sea-birds, living exclusively on fish, should be of interest in this respect. The fats of comparatively few birds have been examined, and those of only one (the hen) quantitatively analysed [Hilditch *et al.* 1934]. The hen is particularly interesting in that its fat contains about 7 % of hexadecenoic acid, until recently considered characteristic of aquatic life. However, hen fat is not closely similar to that of fish, since it contains only traces of C_{20} and C_{22} acids. Koyama [1928] has examined the fats of a number of birds qualitatively, and shown that some (the carnivorous ones) give small yields of ether-insoluble polybromides. In the present paper the results of the quantitative analyses of the body fats of four species of sea-bird are given.

Experimental

The birds were obtained in Shetland waters where it is almost certain that their diet would consist wholly of fish or other aquatic life. The percentages of fat recorded are on the plucked, eviscerated and decapitated birds. Small quantities of accompanying phosphatides (order 10 % of total) were removed by means of acetone. In Table I are given the particulars of the fats and in Table II the compositions.

Table I. *Particulars of fats*

Species	No. of specimens	Fat content %	I.V.	Unsaponifiable %
Herring gull (*Larus argentatus*)	7	9·7	107·9	3·0
Skua gull (*Megalestris catarrhactes*)	7	7·0	95·8	6·6
Gannet (*Sula bassana*)	2	6·7	123·9	8·1
Fulmar petrel (*Fulmarus glacialis*)	6	15·2	130·2	7·8

Table II. *Composition of fats (wt. %)*

Species	Saturated C_{10}	C_{14}	C_{16}	C_{18}	C_{20}
L. argentatus	Nil	3·3	18·5	6·2	0·2
M. catarrhactes	Nil	1·9	16·4	5·7	0·2
S. bassana	Trace	3·2	17·1	3·6	Nil
F. glacialis	0·3	2·0	13·9	3·2	Nil

Species	Unsaturated C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}
L. argentatus	0·5	4·0 (−2·3 H)	30·5 (−2·8 H)	20·3 (−4·1 H)	16·5 (−4·9 H)	Trace?
M. catarrhactes	0·4	4·6 (−2·1 H)	32·6 (−2·6 H)	19·7 (−3·3 H)	18·5 (−3·8 H)	Trace
S. bassana	1·0	5·2 (−2·0 H)	28·3 (−2·8 H)	24·2 (−4·0 H)	17·4 (−6·0 H)	—
F. glacialis	0·9	3·9 (−2·0 H)	26·9 (−2·8 H)	26·8 (−4·0 H)	22·1 (−6·6 H)	Trace?

The small quantity of acid lower than C_{14} in two of the fats was only examined in the case of the fulmar petrel. It appeared to consist of *n*-decoic acid, no lauric acid being present.

Discussion

It can be seen at once from Table II that all four fats are of the "aquatic" type. By comparison with the frequency curves for marine fish fats [Lovern, 1937] it can be seen that these bird fats are also closely similar to the average marine fish fat. Total C_{16}, C_{20} and C_{22} acid percentages roughly coincide with the maxima on the curves. Total C_{18} is rather high, but on the whole it is obvious that the fats of these birds cannot be very different (as regards the proportions of the various acid groups) from the fat which they ingest.

At the same time the fats have been considerably altered in unsaturation. The stearic acid content is higher than in fish fats, whilst in two cases arachidic acid (which can hardly have been ingested) appears. Concurrently the degrees of average unsaturation of the C_{20} and C_{22} acids are reduced below the average for fish fats. Apparently all the birds have hydrogenated the ingested fat, the process having gone further in the two gulls than in the other two birds. The reason for such hydrogenation may be found in the higher temperature of birds than fish. In the case of fish themselves the author has shown [Lovern, 1938] that a difference of 9° has a slight effect of the type expected.

These birds thus form another exception to the broad rule that fat types can be correlated with phylogenetic relationships. The position may perhaps be summarized thus. Many animals, such as the fish discussed by the writer [Lovern, 1937], produce characteristic depot fats considerably different from the fat which they ingest. The differences are specific and can be correlated with the phylogenetic relationships of the animal in question. Certain other animals (including many fish, such as the cod) deposit fat closely resembling that which they habitually ingest. This may mean one of at least two things: (*a*) that they have no specific requirements and any type of depot fat will serve equally well, or (*b*) that in the course of evolution their specific requirements have been produced or modified to suit the normal diet. To decide between these two ideas it would be necessary to make many feeding experiments with different fats and with fat-free diets. Possibly theory (*a*) would apply to some animals and theory (*b*) to others.

SUMMARY

The fats of four species of sea-bird have been quantitatively analysed. They markedly resemble the fats of marine fish in the proportions of the various acid groups, although some hydrogenation has occurred.

These birds form another exception to the broad rule that fat types can be correlated with phylogenetic relationships. Two possible explanations are advanced.

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CCLXXVIII. NOTE ON TWO DERIVATIVES OF OESTRONE

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In connexion with some other investigations we had occasion to prepare *oestrone β-naphthoate* and *oestrone diethylaminoethyl ether*. Neither of these substances appears to have been previously described and we have had them tested for oestrogenic activity. Oestrone α-naphthoate has been prepared by MacCorquodale *et al.* [1936], but no record was made of its oestrogenic activity. Oestrone β-naphthoate resembles the corresponding benzoate [Butenandt, 1930] in that it produces in rats a prolonged period of oestrus although the onset of oestrus is delayed longer than with oestrone itself. The diethylaminoethyl ether of oestrone is of interest as it yields water-soluble salts, but when tested in rats it showed little or no oestrogenic activity.

Experimental

Oestrone β-naphthoate. A solution of oestrone (0·5 g.) and β-naphthoyl chloride (0·6 g.) in pyridine (2·5 ml.) was kept at room temperature for 12 hr. and then heated to 70° for 1 hr. On pouring into a mixture of crushed ice and H_2SO_4 a white solid separated which was collected, washed with aqueous Na_2CO_3, then with water and dried. Extraction with amyl ether gave a small amount of β-naphthoic anhydride and the residue was practically insoluble in diethyl ether. The crude product (0·6 g.) was insoluble in water and glacial acetic acid and very sparingly soluble in alcohol, ethyl acetate and ligroin. Recrystallized from dioxane it had m.p. 262–264°. (Found: C, 81·8; H, 6·4 %. $C_{29}H_{28}O_3$ requires C, 82·1; H, 6·6 %.)

Biological test. In rats doses of 600 and 100γ produced oestrus after 100 hr. lasting for 10 days; 24, 12, 6γ produced oestrus after 100 hr. lasting for 4 days. The threshold dose appeared to be between 1 and 2·5γ.

Oestrone diethylaminoethyl ether. A mixture of oestrone (50 mg.) diethylaminoethyl chloride (120 mg.) and aqueous KOH (25 ml. of 30 %) was heated on the water bath for 2 hr., during which time the oestrone slowly dissolved. After cooling and diluting with water the mixture was filtered. The filtrate acidified with HCl gave a precipitate of oestrone (11 mg.) which was collected, and the mother liquor combined with the solution obtained by dissolving the initial filter residue in dilute HCl. The combined acid solutions were again made alkaline and the solid precipitate collected and recrystallized from dilute alcohol. The diethylaminoethyl ether of oestrone (42 mg.) formed glittering leaflets. m.p. 76–77°. (Found: N, 4·0 %. $C_{24}H_{37}O_2N$ requires N, 3·8 %.) In biological tests it showed no oestrogenic activity in doses of 6, 100 and 1000γ. The base dissolved

in alcohol and treated with ethereal HCl gave the *hydrochloride*, which, crystallized from a mixture of absolute alcohol and ethyl acetate, had M.P. 190–191°.

The biological tests were carried out by Messrs Hoffman-La Roche and Co., Basle, to whom we express our thanks.

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CCLXXIX. THE ORIGIN OF THE PHOSPHORUS COMPOUNDS IN THE EMBRYO OF THE CHICKEN

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From the Institute of Theoretical Physics, University of Copenhagen

(*Received 31 August 1938*)

Several of the numerous compounds containing phosphorus present in the embryo of the chicken[1] occur in the yolk and the white of the egg. Those which do are chiefly phosphatides and nucleoproteins but, as Table I shows, other phosphorus compounds also occur in those parts of the egg.

Table I [Plimmer & Scott, 1909]. *Percentage of the total P (94 mg.) at the beginning and the end of incubation of a hen's egg*

	Beginning	End
Inorganic P	Trace	60
Water-soluble P	6·2	8·6
Ether-soluble P	64·8	19·3
Vitellin-P	27·1	0
Nucleoprotein-P	1·9	12

Kugler [1936] has lately found that, on the twentieth day of incubation, i.e. the last day but one, only 25 mg. of the 65 mg. of lipoid P originally present in the yolk remained there; 8 mg. were found in the embryo, and the remainder had been hydrolysed yielding inorganic P. About two-thirds of the phosphatides present were found to be lecithin and one-third kephalin. In view of the large store of phosphatides present in the yolk even shortly before the egg is hatched, we should expect the embryo to avail itself of this store when it needs phosphatides to build up its nervous system and other organs containing these substances. We can test this point by introducing labelled (radioactive) sodium phosphate into the egg before incubation and investigating if and to what extent the phosphatide of the yolk and of the embryo become labelled. If none becomes labelled, we can conclude that the phosphatide molecules in the embryo are not newly synthesized from inorganic phosphate present there; if, however, the yolk phosphatide remains unlabelled while that of embryo becomes radioactive, we can conclude that the phosphatide molecules present in the embryo have not come from the yolk but have been built up in the embryo with the participation of labelled inorganic P. Similar considerations apply to certain other compounds occurring in the embryo.

Methods

The phosphorus content of a series of solutions is usually determined colorimetrically. For example, the inorganic P present in one sample of an acid-soluble fraction can be determined in this way, and then in another sample the

[1] A detailed investigation of the acid-soluble phosphorus compounds present in the embryo of the chicken was recently carried out by Needham *et al.* [1937].

phosphagen-P present can be converted into inorganic P, so that colorimetric determination now supplies the value for the inorganic P+phosphagen-P. In our experiments this was inadequate. We had to measure not only the P content but also the activity of the various fractions, so we had to obtain precipitates in each case. To obtain sufficient precipitate when dealing with eggs only incubated for a few days, it was necessary to work with several eggs simultaneously.

We precipitated the phosphorus, after bringing it into the inorganic state, as ammonium magnesium phosphate. The precipitate was then dissolved in 0·1 *N* HCl and an aliquot part was sucked into a glass cuvette. This was placed below the Geiger counter used to determine the activity of the preparations, while another aliquot part was utilized for the colorimetric determination of the phosphorus content. The glass cuvettes were covered with a thin mica window (5–6 mg. per cm.^{2}) which only absorbed to a negligible extent the β-rays emitted by the radioactive phosphorus; the area of the mica window was 1·1 cm.^{2} and the liquid content of the cuvette amounted to about 0·5 ml. If we attempt to precipitate ammonium magnesium phosphate from the same solution several times, both before and after hydrolysis, large amounts of salts accumulate in the solution and hinder quantitative precipitation.

We were interested in the determination of the activity of 1 mg. P prepared from different phosphorus compounds present in the embryo or in the remains. Accordingly we were not concerned with the quantitative amounts of the P compounds present and so concentrated our efforts on obtaining the various fractions in a pure state—to avoid, for example, traces of inorganic phosphate remaining in the phosphatides extracted from the yolk. As the phosphatides of the yolk were found to be but slightly active, while the inorganic P was strongly active, even a small contamination of the former by the latter was to be avoided. The white, the yolk, the embryo and, in some cases, the amniotic and allantoic liquids were worked up simultaneously.

As regards the white we were only interested in the total activity present after incubation. The white was ignited (reduced to ash) and the phosphorus in it precipitated as ammonium magnesium phosphate.

The yolk was dried with acetone and the phosphatides extracted three times from the dry product with a 3 : 1 alcohol-ether mixture. The alcohol and ether were then evaporated off at about 50° *in vacuo* and the residue was taken up with light petroleum and filtered. The filtrate was evaporated *in vacuo*, the residue ignited, and the phosphorus precipitated as ammonium magnesium phosphate.

Another part of the yolk was treated as follows. The acid-soluble compounds were extracted, then the phosphatides were removed as described above, and the residual part containing mainly vitellin-P and nucleoprotein-P was ignited; the P content of this last part was determined as ammonium magnesium phosphate.

The embryos were dropped, immediately after being removed from their eggs, into liquid air and were subsequently pulverized. The embryo powder was then extracted several times with cold trichloroacetic acid—in the first two extractions a 10 % solution was used, and later one of 5 %. The extract was filtered into cold concentrated NaOH solution and divided into three parts, (*a*), (*b*) and (*c*). From (*a*) a sample of the average acid-soluble P of the embryo was secured, (*b*) was precipitated with 25 % barium acetate solution at *p*H 6·5. The cold precipitate was washed with a dilute barium acetate solution, centrifuged and dissolved in a few drops of cold HNO_3. The inorganic P present was then precipitated by adding Fiske's reagent. The remaining filtrate was hydrolysed with *N* HCl at 100° for 7 min. to split the two labile phosphate radicals of

adenosinetriphosphoric acid. The phosphorus set free was finally precipitated as ammonium magnesium phosphate. Barium hydroxide was added to the filtrate from the barium precipitation to remove any inorganic P, the precipitate was separated by centrifuging and ethyl alcohol was added to the remaining liquid until an alcohol concentration of nearly 60% was reached. The precipitate obtained after addition of alcohol [Ostern *et al.*, 1936] contained the hexosemonophosphate. Its P content was determined in the usual way. The third part, (*c*), was hydrolysed with *N* HCl and 0·1 *M* ammonium molybdate for 30 min. at 40°. In the course of 30 min. most of the phosphagen present decomposed, so that the inorganic P originally present as such, and that obtained by the decomposition of the phosphagen,[1] were secured together in this fraction.

After removal of the acid-soluble P the embryo was thoroughly treated with an alcohol-ether mixture, as described above, to remove the phosphatides. The residue, containing mainly nucleoprotein-P, was ignited with concentrated sulphuric and nitric acids and the P precipitated in the usual way.

Results

Eggs incubated for 6–18 days. The results of the determination of the specific activities (activities per mg. P) of the different fractions extracted from seven embryos and from the remaining parts of eggs incubated for 11 days are shown in Table II, while Tables III–V give the results obtained with eggs incubated for 18, 16 and 6 days. In addition to the specific activity (activity per mg. P, with that of the P extracted from the white of the egg taken as 100), we have also recorded in Tables II and III the activity (in kicks per minute or in % of amount injected) and the P content of the fraction—this last quantity being determined, in all cases, by the method of Fiske & Subbarow.

Table II. *Specific activity of P extracted from different fractions of an egg incubated for 11 days. (Specific activity of P extracted from the white taken as 100)*

	Fraction	mg. P*	Kicks per min.	Specific activity
Embryo:	Average acid-soluble P	0·074	3·5	59
	Inorganic P	0·077	3·1	51
	Adenosine-P + inorganic P	0·121	6·0	63
	Creatine-P	0·171	8·1	60
	Phosphatide-P	0·561	29·6	67
	Residual ("nucleoprotein") P	1·49	85·6	72
Yolk:	Phosphatide-P	10·4	0·55	0·067

* Indicator mg. P in the sample measured.

Table III. *Specific activity of P extracted from different fractions of an egg incubated for 18 days. (Specific activity of P extracted from the white taken as 100)*

	Fraction	mg. P	% of amount injected	Specific activity
Embryo:	Average acid-soluble P	19·7	53·5	19
	Inorganic (without skeleton) P	10·91	27·2	17
	Tibia and femur-P	4·50	7·6	11
	Adenosine-P	0·048	0·14	20
	Phosphatide-P	1·08	1·7	11
	Residual ("nucleoprotein") P	0·204	0·3	10
Yolk:	Acid-soluble P	0·828	1·3	11
	Phosphatide-P	17·50	0·28	0·11
	Residual P	2·16	0·12	0·40

[1] On the phosphagen content of the embryo of the chicken, cp. Lehmann & Needham [1937].

The figures for the specific activities (activities per mg. P) of different fractions extracted from an embryo and from the remaining parts of an egg incubated for 18 days are shown in Table III. The P content in mg., the percentage of the injected activity present in the fraction and the relative specific activity are recorded; the specific activity of the P extracted from the white of the egg is taken as 100.

The specific activities obtained when the eggs were incubated for 16 and 6 days respectively are seen in Tables IV and V.

Table IV. *Specific activity of P extracted from different fractions of an egg incubated for 16 days. (The specific activity of P extracted from the white taken as 100)*

	Fraction	Specific activity
Embryo:	Average acid-soluble P	14
	Inorganic (without skeleton) P	14
	Tibia and femur-P	15
	Creatine-P	14
	Hexosemonophosphate-P	19
	Phosphatide-P	12
	Residual ("nucleoprotein") P	16
Yolk:	Acid-soluble P	12
	Phosphatide-P	0·14
	Residual P	1·22

Table V. *Specific activity of P extracted from different fractions of 10 eggs incubated for 6 days. (Specific activity of embryo phosphatide P taken as 100)*

	Fraction	Specific activity
Embryo:	Phosphatide P	100
	Average (phosphatide) P	113
Yolk:	Inorganic P	60
	Acid-soluble minus inorganic P	34
	Phosphatide P	0·032
	Residual P	1·3

As the figures show, the phosphatides extracted from the yolk are only slightly active, while those extracted from the embryo show strong activity; 1 mg. of embryo phosphatide-P is at least 100 times as active as 1 mg. yolk phosphatide P. Furthermore, the specific activity of the embryo phosphatide-P is about as high as that of the embryo inorganic P, showing that an inorganic P atom reaching the embryo has about the same chance of entering the skeleton as of being incorporated in a phosphatide molecule by an enzymic process—which of the two systems it enters is governed solely by probability considerations. From this it follows that the phosphatide molecules in the embryo are not identical with those derived from the yolk, but are synthesized in the embryo.

The formation of labelled phosphatides in growing eggs was investigated by Hevesy & Hahn [1938]. It was found that the phosphatides present in the yolk are taken up from the plasma by the ovary and incorporated into the latter; as soon as the yolk leaves the ovary no more change occurs in the content or composition of its phosphatides. When labelled phosphate is administered to a hen after the yolk has left the ovary and is located in the oviduct, the egg takes up active phosphate but no active phosphatide is formed. In experiments *in vitro* as well, eggs placed in radioactive sodium phosphate solution take up active phosphate but no active phosphatides are formed. The slight activity of the phosphatides present in the yolk of incubated eggs is presumably due to the

influx into the yolk of small amounts of active phosphatides synthesized in the embryo. This view is supported by the fact that the ratio of the specific activities of the embryo phosphatide-P and yolk phosphatide-P was much larger (3000) in the 6 days experiment than in the 18 days experiment (100). The activity of the residual P of the yolk, which is mainly composed of vitellin and nucleoprotein, was larger than that of the phosphatides; this can be understood if we admit the possibility that the extraction of the strongly active, non-protein constituent of the yolk is not quantitative, for in this case the specific activity of the residual P would be increased.

The embryonic residue obtained after extraction of the acid-soluble and ether-soluble constituents is composed chiefly of nucleoproteins. That the specific activity of the nucleoprotein-P is the same as that of the inorganic P extracted from the embryo is not surprising, because much less nucleoprotein is present in the yolk than in the embryo (Table I). The greater part of the nucleoproteins present in the embryo must therefore have been built up in the course of incubation; during this process labelled phosphate has an opportunity of entering the nucleoprotein molecules.

The radioactive sodium phosphate of negligible weight injected into the white of the egg labels the inorganic P present in the latter. The labelled inorganic P is transported from place to place, along with the other water-soluble phosphorus compounds present in the white and the yolk, and becomes partly incorporated in the embryo. The inorganic P so removed is replaced by some formed by hydrolysis, mainly of phosphatides and vitellin. In this way the radioactive inorganic P injected into the egg becomes more and more diluted with inactive inorganic P and its specific activity (activity per mg. P) diminishes accordingly. Whereas at the start of the experiment only traces of inorganic P are present in the egg, at the end of the incubation about 60 mg. are found, mostly in the skeletal part of the embryo. As the inorganic P used in the synthesis of the different P compounds present in the embryo becomes less and less active in the course of incubation, we should expect to find the phosphorus compounds synthesized at a later date much less active than those built up at an early stage in the incubation. This does not, however, seem to be the case. Actually, we have not yet compared the activity of, for example, the phosphatides extracted from the nervous system, which is built up at an early stage, with that of the phosphatides extracted from the skeleton, which is synthesized at a late stage of development. But the fact that the specific activities of the inorganic P, the phosphatide-P and the nucleoprotein-P were found to be equal to within the errors of the experiment suggests that no large differences in the specific activities of phosphorus compounds formed at different dates can be expected, though minor differences could possibly be found. This must be interpreted as being due to the ceaseless breaking-up and the rebuilding of the molecules in the embryo under enzymic action, a process which leads to equipartition of the activity between the different phosphorus molecules.

We should most expect to find phosphate layers of different specific activity in the skeleton. The rate of phosphorus exchange in the bone tissue is a comparatively slow process, though the atoms of the embryonic bone tissue may be comparatively easily replaceable. A difference in the specific activities of inorganic P extracted from bone tissue (tibia + femur) and from the other organs of the embryo is shown in Tables III and IV, which is, however, to be interpreted cautiously.

Distribution of radioactive phosphate in the egg

The greater part of the sodium phosphate injected into the white is still found at the end of the experiment in that part of the egg. The distribution of the activity between white, yolk, connecting fluids (which were not, however, free from white and yolk) and embryo is seen in Table VI.

Table VI. *Distribution of injected active phosphate between different parts of the egg*

Time of incubation	Fraction	% activity
6 days	White	61·6
	Yolk	10·3
	Liquids	26·0
	Embryo	1·7
18 days	White	14·9
	Yolk	1·7
	Liquids	19·8
	Embryo	63·0

The low activity of the yolk might possibly be due to a slow rate of penetration of the vitellin membrane by the phosphate ions; this point is under investigation. Another possible explanation is that the inorganic P content of the yolk is lower than that of the white. If a distribution equilibrium is reached, the activity should be proportional to the amount of inorganic phosphate present in the phase in question, since the inorganic P, among all the P compounds present in the yolk and white, is practically the only source of activity; in the 6 days experiment, for example, 10% of the 10·3% activity found in the yolk was present as inorganic P. Finally we have to envisage the possibility that a part of the inorganic phosphate injected is not freely movable in the white—it might be precipitated as calcium phosphate or attached to proteins, its mobility being lowered thereby.

We have also carried out experiments in which 0·1 ml. physiological NaCl solution containing a negligible amount of labelled sodium phosphate was injected into eggs which were not incubated. After the lapse of 5 days the distribution of the activity in different parts of the egg was determined; 97% was found in the white and 3% in the yolk. As was of course to be expected, a still greater preference for the white was shown by the active phosphorus in this experiment; the duration of the experiment was shorter than that of those discussed above, and transport of phosphorus from the white to the embryo was absent.

To test whether the water injected encountered any hindrance in its propagation through the egg, we injected 0·2 ml. heavy water into the white of the egg; after the lapse of 5 days water was distilled separately from the white and from the yolk and the densities determined. We are much indebted to Mr O. Jacobson for carrying out the density determinations using Linderström-Lang's float method. He found that the water prepared from the white had a density exceeding that of normal water by 484 parts per million, while the corresponding figure for the water obtained from the yolk was 437. The deuterium content of the water distilled off from the yolk was thus found to be only about 10% lower than that of the water from the white, showing that in the course of 5 days the water injected was very nearly evenly distributed throughout the egg, in contrast to the injected active phosphate. The anomalous behaviour of the latter, while of interest in the study of the circulation of phosphate ions in white and yolk, in

no way influences the investigation of the main problem discussed in this paper—namely, if and to what extent the molecules of the different phosphorus compounds present in the embryo are built up there or drawn, ready made, from the yolk.

Introduction of labelled hexosemonophosphate into the egg to be incubated

In one set of experiments, instead of following up the fate of labelled inorganic P in incubated eggs, we introduced radioactive hexosemonophosphate. Prof. Parnas very kindly presented us with this radical (prepared by Dr Ostern) in the form of barium hexosemonophosphate, from which, by treatment with sodium sulphate in the cold, the sodium compound of the ester was obtained. 0·2 ml., containing about 0·2 mg. P as hexosemonophosphate salt and about 3 mg. sodium sulphate, was injected into the white of each of the eggs to be incubated; to avoid decomposition of the ester, the solution was kept ice-cooled until it was injected into the egg. Of the 10 eggs receiving this treatment, only two supplied living embryos. After a lapse of 14 days, 7·7 % of the activity injected was found to have been incorporated in the embryo (5·8 % in the yolk) and a large fraction was also to be found in the white and in the connecting liquids. If, of the various fractions extracted from the embryo, we had only found activity in the fraction containing hexosemonophosphate, we should have had to conclude that the hexosemonophosphate does not decompose in the egg but enters the embryo as such. In view of the results obtained in the experiments carried out with labelled inorganic phosphate, however, such behaviour was hardly to be expected. Furthermore, Kay [1926] found that in the embryo the phosphatase activity of the developing bone was extremely high, the phosphatase decomposing the hexosemonophosphate. We isolated the hexosemonophosphate from the embryo, as described on p. 2148, and compared the specific activity of this fraction with that of the inorganic phosphate (+creatine-P). We also isolated the phosphatide fraction and the residual phosphorus fraction containing mainly nucleoprotein-P. As Table VII shows, no conspicuous difference can be seen between the specific activities of the different fractions of the embryo, with the possible exception of the residual P. In these experiments small activities had to be measured and the differences found between the first three fractions lie within the errors of the experiment. The results obtained suggest the explanation that active inorganic P splits off from the labelled hexosemonophosphate injected and is incorporated in the different phosphorus compounds of the embryo, while the hexosemonophosphate molecules extracted from the embryo are not those synthesized by Dr Ostern but are molecules built up by the chicken's embryo.

Table VII. *Specific activity of P from different fractions extracted from two eggs incubated for 14 days after the injection of radioactive hexosemonophosphate. (Specific activity of P extracted from the white taken as 100)*

	Fraction	Specific activity
Embryo:	Inorganic P	24
	Hexosemonophosphate-P	26
	Phosphatide-P	20
	Residual ("nucleoprotein") P	11
Yolk:	Inorganic P	36
	Hexosemonophosphate-P	18
	Phosphatide + residual P	0

The low value found for the residual P of the embryo may possibly be due to the building up of a part of the nucleoprotein fraction at an early date before much of the active hexosemonophosphate introduced has decomposed. The phosphatide-P and residual P extracted from the yolk were found to be inactive. These fractions were found to be only slightly active even after the injection of strongly active inorganic P, and the absence of activity after the injection into the egg of the much weaker hexosemonophosphate was only to be expected. The hexosemonophosphate fraction isolated from the yolk had a specific activity of 18; the inorganic P, 36. The larger value found for the specific activity of the inorganic P is possibly to be explained in the following way. Some active hexosemonophosphate diffuses into the yolk and partly decomposes into active inorganic P; this is the source of most of the active inorganic P which we isolated from the yolk. The hexosemonophosphate, isolated by the method outlined on p. 2149, contains, besides the active hexosemonophosphate, some non-active hexosemonophosphate and possibly also some other acid-soluble P compound separated simultaneously, which diminishes the specific activity of the "hexosemonophosphate" fraction isolated from the yolk. In the embryo, on account of the strong enzymic action prevailing there, all phosphorus compounds become activated; on the other hand, in the yolk, as we have just mentioned, no such activation takes place.

On the phosphatide synthesis in the embryo of the chicken

We saw that the phosphatide molecules present in the chicken's embryo are not identical with those formerly located in the yolk, but that they were synthesized in the embryo. The work of Schönheimer & Rittenberg [1936] gives us important information about the units which are utilized in the synthesis. They found, by making use of deuterium as an indicator, that the developing hen's egg forms no new fatty acids and their result excluded also the possibility that unsaturated fatty acids present in the egg had been hydrogenated during development. Needham [1931], on the other hand, found that a marked desaturation occurs in an aqueous emulsion of embryonic tissues mixed with the corresponding yolk and vigorously shaken. The embryo must thus make use of the fatty acids present in the yolk to build up its phosphatides; in doing this it possibly gives some preference to the less saturated fatty acids. The fatty acid components of the phosphatides extracted from the embryo are found to be less saturated than those extracted from the yolk residue. The at first sight puzzling fact that the embryo, instead of using the phosphatide molecules found in great abundance in the yolk, synthesizes its own phosphatide molecules, becomes less puzzling when we envisage the likely possibility that the synthesis of phosphatide molecules is also a step in other chemical processes, which occur simultaneously in the growing embryo.

SUMMARY

Radioactive sodium phosphate was injected into hen's eggs which were then incubated in some experiments for 6, and in others for 11, 16 and 18 days. While the phosphatide-phosphorus extracted from the embryo always showed a high specific activity (activity per mg. P), that extracted from the yolk was hardly active at all. The phosphatide molecules present in the embryo could not therefore have been taken from the yolk only, but must have been synthesized in the embryo. The investigation of the "acid-soluble" and residual (mainly nucleoprotein) phosphorus extracted from the embryo led to a similar

result—namely, that the ratio in which the labelled inorganic phosphorus atoms are incorporated into the different phosphorus compounds present in the embryo is governed solely by probability considerations. Practically all the phosphorus atoms present in the various compounds of the embryo must pass through the stage of inorganic P; only the inorganic phosphorus present in the embryo is taken as such from the yolk or the white.

In some experiments, instead of radioactive sodium phosphate, labelled hexosemonophosphate was injected into the egg before incubation. The hexosemonophosphate-phosphorus extracted from the embryo had about the same specific activity as the inorganic and the phosphatide phosphorus extracted. This result suggests that inorganic phosphate radicals which have split off from the hexosemonophosphate and from other compounds present in the yolk and the white, rather than the hexosemonophosphate molecules introduced into the latter, are utilized to build up the phosphorus compounds of the chicken's embryo.

We wish to express our best thanks to Prof. N. Bohr for the numerous facilities kindly placed at our disposal and to the Danish State Farm at Hilleröd for incubating many of the eggs investigated.

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CCLXXX. THE VITAMIN B_1 CONTENT OF FOODS
II. ADDITIONAL VALUES

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We have previously published the vitamin B_1 values of about 80 foods [Baker & Wright, 1935]. The following additional values have been obtained by the same technique. A few figures for breads, some of which have been published elsewhere [Baker *et al.* 1937], are included for convenience of reference. Values are given in terms of International units.

Material	Dose (g.)	I.U./g.	Remarks
		Meat, offal and fish	
Raw lean beef	4	0·3 –0·5	0·5 previously published
	6	0·4	
Cooked tongue	4	0·3 –0·45	—
Stewed steak	6	0·1 –0·2	—
Stewed rabbit	4	0·2 –0·4	Same sample
	6	0·2	
Stewed tripe	6	Less than 0·2	—
Fried sweetbread	4	0·2–0·4	—
Fried bacon	0·5	2·8 –4·8	Cf. roast pork 3·2, boiled ham 2·2
Fried herring	4	0	—
		Dairy produce	
Sweetened condensed milk	5	0·4 –0·6	Cf. raw milk 0·23
		Vegetables	
Cooked haricot beans	4	0·4 –0·6	Cf. raw 1·2
	6	0·35–0·6	
Cooked butter beans	4	0·2 –0·3	—
Raw green runner beans	4	0·25–0·75	—
Raw cauliflower	4	0·4 –0·5	Previous value 1·1
Raw marrow	8	0·1 –0·2	—
Raw fresh peas	1	1·6 –2·8	—
Cooked fresh peas	4	0·8 –1·1	Same sample
	2	1·0 –1·2	
Raw dried peas	1	4·2 –4·8	Cf. canned peas 1·2
Cooked dried peas	3	0·15–0·45	—
	5	0·2 –0·4	
Stewed onions	4	0·3 –0·5	Cf. raw 0·4
		Fruits	
Black currants	5	0·0 –0·2	—
Blackberries	5	0·0 –0·2	—
Melon	10	0·1 –0·2	—
Fresh ripe peach	4	0·1 –0·4	—
Canned peach	5	0·1 –0·3	—
Syrup from peach	—	Traces only	—
Raspberries	6	0·25–0·35	—
Red currants	5	0·1 –0·2	—
Strawberries	4	Traces only	—
		Cereals	
Boiled rice	8	—	—
Ground rice	8	0·15–0·35	—
Sago	8	—	—
Tapioca	8	—	—

Material	Dose (g.)	I.U./g.	Remarks
		Cereals (*cont.*)	
Semolina (1)	8	0·35–0·45	2 different samples
(2)	4	0·55–0·65	
Millet (Juba)	3	0·6 –1·0	—
	2	0·8 –1·2	
Oatmeal	1	1·1 –1·7	Previous figures 3·25, 1·4
	2	1·0 –1·2	
Rolled oats	1	1·8 –2·7	—
	2	1·45–2·0	
		Miscellaneous	
Black treacle W. Indian	1	—	—
		Breads	
Special brown (London):			
Sample (1)	3	0·45–0·6	Cf. previous values of other brown breads, 0·5, 0·7, 0·5
Sample (2)	3	0·6 –1·0	Cf. previous values for wholemeal bread, 1·1, 1·2, 1·2
Special brown (Cheshire)	3	1·2 –1·4	Probably in the germ bread class. Cf. previous values of other germ breads, 1·2, 1·65, 1·7
Wholemeal stone-ground	4	0·4 –0·6	A bread definitely lower in vit. B_1
(Cambridge)	3	0·35–0·45	than others known to be wholemeal
Austrian, Sample (1)	3	0·5 –0·7	Cf. also previously published
Sample (2)	3	0·5 –0·9	values for white bread; 4 samples London 0·14, 0·14, 0·15, 2·0; 1 sample Scotland 0·24; white with malt, 2 samples, 0·35, 0·35

These foods have all been assayed by the rat-bradycardia method of Birch & Harris [1934], and since this method has been in use in this laboratory for 4 years a few comments may be useful.

Values obtained are in good agreement with those published by other workers, e.g.:

Material	Value by bradycardia method I.U./g.	Values reported by other workers, I.U./g.
White bread	0·14–0·2	0·12–0·15 [Harris, 1937] 0·17–0·25 [Copping & Roscoe, 1937]
Brown bread	0·5 –0·7	0·6 [Morgan & Frederick, 1935, 1]
Whole wheat bread	1·15, 1·2	1·17, 1·3 [Morgan & Frederick, 1935, 1]
Wheat, whole grain	1·2 –3·4	1·5 [Scheunert & Schieblich, 1937]
Bran	1·3, 3·0	3·6 [Leong & Harris, 1937]
Wheat germ, raw	4–22 (118 samples)	7·6–9·4 [Morgan & Frederick, 1935, 2]
Brewer's yeast	6·0 –23	16–18 [Chick & Jackson, 1932] 18–20 [Birch & Harris, 1934]
Banana	0·5	0·3–0·6 [Van Veen, 1935]
Potato	0·4	0·3–0·6 [Van Veen, 1935]
Lean beef	0·5	0·3–1·0 [Van Veen, 1935]
Raisins	0·75	0·5 [Morgan *et al.* 1935]

Assays repeated after long intervals have been found to give consistent results, e.g.:

	I.U./g.
White bread, sample (1) assayed 1935	0·0–0·2
(2) assayed 1936	0·1–0·2
(3) assayed 1937	0·1–0·3
Whole wheat, 1 sample, 1st assay 1935	1·6–2·4
2nd assay 1936	1·9–2·1

Recently Robertson & Doyle [1937] have called attention to various difficulties in the use of the method. They find wide differences in the rats' heart rate as recorded at two portions of the same electrocardiographic tracing and also occurring after a short interval without dosage of any vitamin B_1. As the result

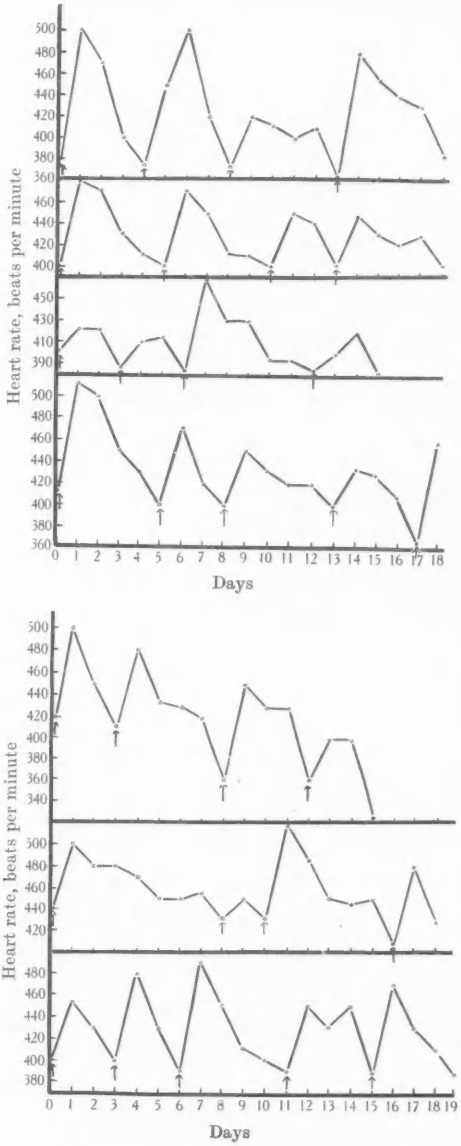

Fig. 1. Heart rates recorded daily. Seven rats taken consecutively from the record book.

of experience involving over 80,000 readings, we do not find these difficulties serious. Irregularities such as extra systoles and dropped beats occur sometimes, though not often, and in these cases it is important to note that it is the rhythm which is being counted, not the actual number of beats. In electrocardiographic tracings 8–10 in. long, we do not find a variation greater than 5–10 beats per min., representing 1–3% of the total heart rate and only a fraction of the variation which is brought about by an increased intake of vitamin B_1.

The accompanying graphs (Fig. 1) are records of the behaviour of a group of 7 consecutive rats taken from the daily record book. These show that the heart rate after a dose—as seen in 24-hourly readings—rises steeply and gradually drops to the initial level.

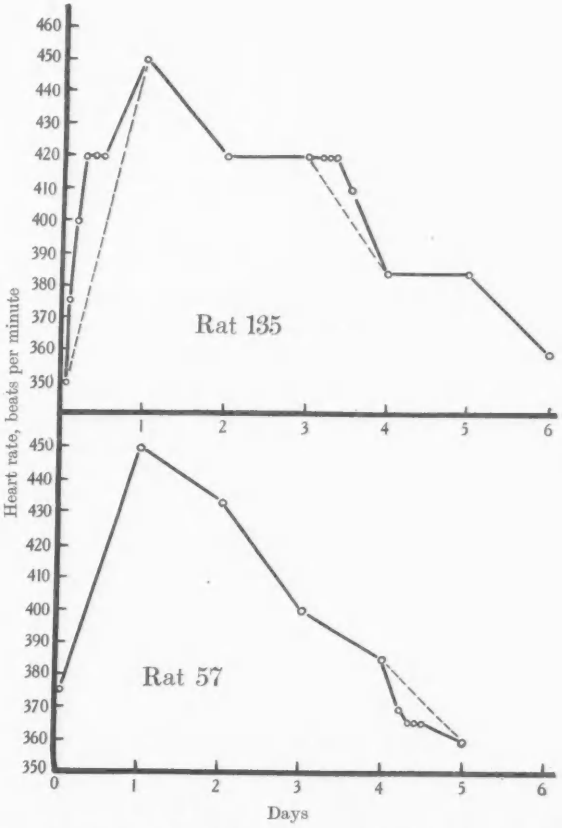

Fig. 2. Records of the heart rates of 2 rats measured 24-hourly and also at more frequent intervals to show the progressive rise or fall in heart rate. —o—o— All readings. – – – 24-hour readings.

The records of rats nos. 135 and 57 (Fig. 2) show further that readings taken at more frequent intervals fall into place with the 24-hourly ones.

The rats' heart rate is, however, sensitive to very small variations in vitamin B_1 intake, and, while this is an advantage in permitting the assay of substances containing traces of the vitamin, it necessitates stringent care in practice to keep a continuous check on the basal diet by having 2 or 3 animals always as negative controls. That this is necessary is shown by the fact that a yeast sold commercially in America for use in vitamin B_1-deficient diets, and guaranteed to be free from vitamin B_1, was found electrocardiographically to contain about 1 I.U. vitamin B_1 per g. This amount in the yeast of a basal diet had actually been the cause of some puzzling results which were later explained when the presence of vitamin B_1 was known. On a basal diet completely devoid of vitamin B_1, the heart rate of a rat, if already dropping, becomes slower every day until death occurs with a rate usually of less than 300 beats per min. (see negative control graph, Fig. 3).

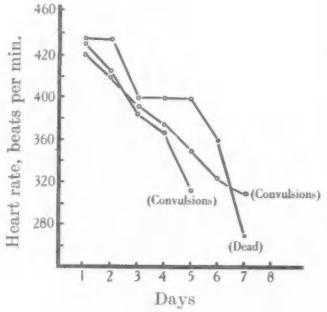

Fig. 3.

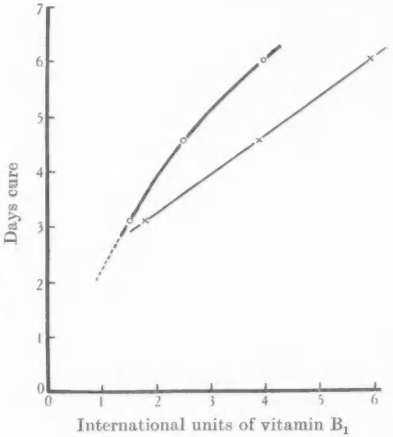

Fig. 4.

Fig. 3. Negative control records.

Fig. 4. The cardiographic response of the rat's heart to International Standard vitamin B_1. Composite curve from over 260 readings at each of 3 dose levels. o—o Duration of cure/dose. ×—× Duration of cure/log dose.

15 mg. Int. St.	25 mg. Int. St.	40 mg. Int. St.
3·1 days (283 readings)	4·6 days (280 readings)	6·0 days (260 readings)

As in all biological assays, it is necessary to test the unknowns simultaneously with the standard of reference. In this laboratory three levels of international standard have been fed weekly and from the results obtained a considerable amount of information has been collected concerning the response to this substance. The curve shown in Fig. 4 was obtained from over 260 readings at each level and is logarithmic in form. There is little difference between the curves obtained from weekly readings and this composite curve, a fact which emphasizes the specificity of the cardiac response to vitamin B_1 and its independence of disturbing factors.

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CCLXXXI. STUDIES OF THE ESSENTIAL UNSATURATED FATTY ACIDS IN THEIR RELATION TO THE FAT-DEFICIENCY DISEASE OF RATS

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The experiments of Burr & Burr [1929; 1930] and of Burr *et al.* [1932] clearly established that the complete exclusion of fat from a diet containing all known accessory factors slowly produced in young rats a definite disease. Its symptoms were cessation of increase in length and weight with development of a dry scurfy skin, scaly tail, irregular ovulation and lesions of the kidney and urinary tract. The rats receiving the fat-free diet ate as much food and drank twice as much water as their controls, but notwithstanding became extremely emaciated. At 4 months after weaning their weight reached a level of about 150 g. and then remained stationary, but weight increase was resumed when 10 drops daily of the fatty acids from lard were added to the diet. The curative effect of the dose was immediate and the gain in weight was quite disproportionate to the calorie value of the curative material administered. Various other oils were tested and the conclusion was drawn that their curative potencies appeared to be correlated with their linoleic acid contents. Burr and Burr therefore suggested that the rat is unable to synthesize linoleic acid and that this acid is essential for its normal growth. Linolenic acid was also examined and was reported to be equal to linoleic acid in curative effect. The full literature of this subject has recently been reviewed by Anderson & Williams [1937] to whose excellent account the reader is referred. Work which is particularly relevant to the present study will be referred to as the various materials tested are discussed.

There is no evidence as to the part played in metabolism by these unsaturated fatty acids. Linoleic acid is present chiefly in the fatty acids of the phospholipins and to a less extent in the neutral fat. Linolenic acid, on the other hand, appears to be changed immediately on entering the body and is not found even in small amounts, unless when large quantities have been administered. It is known, owing chiefly to the work of Sinclair [1929–30], that the highly unsaturated acids are held in the phospholipins with extreme tenacity and are eliminated only very slowly when animals are transferred to a diet from which the acids are absent. They do not appear to represent stages in the combustion of the fatty acids, but to have some unknown significance in the body metabolism. It seemed possible that an oxidation product might be formed as a further stage in some metabolic process and it appeared desirable to compare the effect of the unsaturated acids with that of some closely related oxidation products.

The present investigation has, therefore, included the following:

(1) A reproduction of the work of Burr and Burr with a study, as far as possible quantitative, of the relative potencies of linseed oil, linoleic and linolenic

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acids, both to restore weight increase and to alleviate skin lesions: a test of the potencies of the elaidized linoleic and linolenic acids compared with those of the original products.

(2) A test of the potencies of certain oxidation products of linoleic and linolenic acids.

(3) A comparison of the activities of fractions of the unsaturated acids from lard and from linseed oil.

(4) A comparison of the activities of linseed oil and raisin seed oil.

(5) An examination of the potency of the methyl ester of the highly unsaturated docosahexaenoic acid ($C_{22}H_{32}O_2$) of cod liver oil. Through the generosity of Dr E. H. Farmer an opportunity was afforded to test the efficacy of this acid recently isolated by him from cod liver oil [Farmer & Van den Heuvel, 1938, 2].

(6) A test of the curative actions, if any, of chaulmoogra oil and chaulmoogric acid, of methyl arachidate and of 9:10:12-trihydroxystearic acid.

Biological technique

The experiments described in the present paper have been in progress for about 5 years so that the technique has varied slightly over this period, but the regime of Burr *et al.* [1932] has been followed fairly closely.

Young rats weaned on a stock diet at an age of 21 days and a weight of about 40 g. were placed in separate cages with coarse wire grids. Sometimes fine wire grids and cotton-wool bedding were given when the animals seemed in a precarious condition in severe weather. As it takes months to prepare the animals it is very important not to lose them when the time for testing approaches.

The diet consisted of: casein (British Drug Houses, fat- and vitamin-free), 12 %; sucrose, 84·1 %; salt mixture, McCollum 185 [McCollum & Davis, 1914], 3·9 %.

The diet was given *ad libitum*, slightly moistened. The vitamin B complex was supplied as 0·65 g. per head daily of dried brewer's yeast which had been continuously extracted with ether for 24 hr. This supplement was given separately and was frequently refused at first, but if it was withheld for the first 7–10 days it was subsequently taken with avidity and was found an admirable vehicle for administering the test dose, whether this took the form of an oil or a solid. Vitamins A and D were given as 0·5 mg. daily per head of the unsaponifiable fraction of a cod liver oil concentrate, of value 4000 blue units, dissolved in paraffin oil. In the latest experiments (rats belonging to litters 4273–4350) vitamin A was derived from a fish liver oil concentrate, but vitamin D was given as 2–3 I.U. daily of irradiated ergosterol dissolved as before in paraffin oil. Vitamin E was not given except in the latest experiments (rats numbered as above) when it was given for a portion of the depletion period and for the test period as an unsaponifiable fraction of wheat germ oil, prepared by the Glaxo Laboratories. It was administered without solvent in a dose equivalent to about 2·0 g. wheat germ oil weekly. The distilled water used for drinking and for moistening the food contained 0·27 mg. KI per l.

On this diet the behaviour of the rats conformed with that described by Burr & Burr. After about 2 months the weight curve began to flatten and the skin lesions began to appear and to become progressively more severe. Tests were usually started after about 4 months on the diet, but no rigid rule was established in this respect. A few tests which were started when the rats had only been receiving the deficient diet for about 70 days could not be regarded as reliable, at

least from the standpoint of resumption of weight increase. After a period of 4 months or more on the deficient diet the writers considered that weight increase could be accepted as some measure of the potency of the materials administered and the total weight increase in an experimental period of 35 days was taken as one criterion. The total weight increase was calculated as the weight on the day on which dosing was begun subtracted from the weight 35 days later.

For quantitative estimation, weight increase has been the chief criterion used by Burr and his co-workers, by Moore [1937] and by Turpeinen [1938]. The last also used as criterion the restoration of the oestrous cycle towards normal.

While accepting weight increase as one criterion, the present workers hold very strongly the opinion that, since weight increase is a non-specific response, it should not be used as the only criterion, and that cure of symptoms must be included as an indispensable part of the test. A serious objection to the use of this additional criterion is the great irregularity with which most of the lesions tend to appear. The kidney symptoms described by Burr & Burr were sometimes seen. The development of the so-called scaly tail, better described as a corrugated or annulated tail, was very irregular, the tails of individual rats sometimes being almost normal at a time when their litter-mates were showing a very severe degree of annulation. The other symptoms observed were general dryness of the skin and thinness of the hair with much scurf, showing best on parts of the body where the fur was dark. Dryness and scurfiness of the fore and hind paws and ears were also present; the most regularly observed site for this state was the dorsal surface of the hind feet and front of the ankles. Dryness here occurred invariably and was finally adopted as the most satisfactory criterion for studying the cure of skin symptoms. With potently curative materials the skin of the insteps could be restored to normal within the 35 days of the experimental period during which curative material was administered and weight increase measured. Only three degrees of healing could be satisfactorily distinguished: complete healing, designated by $++$ in the Tables, in which the ankles were restored to normal; partial healing, designated by $+$, in which improvement was definitely present but the ankles were still abnormal; no healing, designated by θ, in which the ankles were unchanged or worse.

Even when lesions of the tail were well developed, as they usually, but not always, were, it was found impossible to use them to measure the rate of healing since the process was much too slow. Within the experimental period of 35 days, even when healing of the ankles was complete, the tail might show little change. If the cure was continued long enough the tail could be restored entirely to normal, but in 35 days it might even appear worse owing to the vigorous desquamation which sets in at the beginning of the healing process. The writers share the impression expressed by Brown & Burr [1936] that the skin lesions are somewhat influenced by weather changes, being aggravated by the same type of cold dry weather as causes the human skin to chap. This adds to the difficulties in appraising cure. Very severe lesions of the external skin about the mouth of the rats were sometimes observed but these were due to the burning action of the curative materials administered and not to the deficiency. For instance, methyl linoleate and linolenate, and the methyl docosahexaenoate in these experiments, and ethyl laurate and acids from hydrogenated tung oil in other experiments, had the effect of seriously damaging the skin about the mouth, causing the upper layers with all the hair attached to shell off. No internal lesions appeared to have been produced. Materials having this effect were therefore always intimately mixed with the dried yeast dose, and in this way did not wet the rat's lips and were not objected to.

Although a source of vitamin E was given in some experiments and not in others there was no convincing evidence that its absence made any significant difference in this test. Skin lesions were healed and weight increase appeared to be promoted as well in its presence as in its absence. A quite different type of skin lesion with large raw and bleeding areas appeared in some cases and may have been due to vitamin E deficiency but this is quite uncertain. All the rats tended to become very nervous and fidgety when untreated and to improve on treatment, so that the essential unsaturated fatty acid was the probable sedative factor.

Since there is no recognized standard material and no curve of response available for tests on the unsaturated fatty acids, estimations of the potency of materials were made as a series of simultaneous comparisons in which the weight increase and degree of healing of the skin of the ankles of litter-mate rats were compared. Some negative controls and some positive controls receiving linseed oil were usually included, but the larger number of rats was devoted to some special comparison. Thus, for example, in the first experiment the action of methyl linoleate was directly compared with that of methyl linolenate; in the second the potencies of linusic and *iso*linusic acids were compared with one another and with that of linseed oil. When only small amounts of material were available or when preliminary experiments gave an unequivocally negative result, full comparisons were not made. A certain number of materials tested in this way are included in Table III.

Since rats for this test take so long to prepare they were sometimes used a second time when the material first tested had proved negative. Negative controls were also subsequently used for tests. As an additional form of positive control, rats which had given a negative result were usually eventually treated with some known positive material such as wheat germ oil or linseed oil, but no record of this form of positive control is included here.

Experimental

(1) *Comparison of the relative potencies of methyl linoleate, methyl linolenate and linseed oil with one test of methyl β-linoleate. Effect of elaidization on the potency of linoleic and linolenic acids.*

Burr *et al.* [1932] found methyl linoleate and methyl linolenate equally effective in curing the lesions and restoring weight increase in rats which had received a fat-free diet. The curative effect of linoleic acid has been repeatedly confirmed [Evans & Lepkovsky, 1932; Tange, 1932; Becker, 1934; Becker, 1935; Moore, 1937; Turpeinen, 1938]. As regards linolenic acid the facts are not so well established. The experiments with pure methyl linolenate seem to have been repeated only by Tange [1932], who concluded that linoleic and linolenic acids were equally effective in promoting weight increase and bringing about a cure. His experiments do not, however, appear entirely to justify this conclusion. He describes experiments in which groups of 2 and 3 rats were dosed daily with one drop of methyl linoleate and linolenate respectively, and says that when growth was somewhat retarded the dose of methyl linolenate was increased to 2 drops but no great improvement was observed; he further reports that this retardation and irregularity, which were not shown with linoleic acid, seemed to be rather influenced by heat. A chart shows as equal the growth curves of two rats dosed with methyl linoleate and of two dosed with methyl linolenate, but no details are given as to the size of the doses.

Since linolenic acid is always accompanied in plant oils by linoleic acid,

it is essential that the linolenic hexabromide from which the linolenic acid is prepared should be carefully purified. In the experiments of Burr & Burr the samples of linoleic and linolenic acids used were obtained from maize and linseed oils respectively. The unsaturated acids were brominated and the resulting tetra- and hexa-bromostearic acids were carefully purified by recrystallization. These were then debrominated, esterified and distilled at less than 1 mm. pressure. The preparation of linolenic hexabromide melted at 180 to 181°, so that it could not have contained any appreciable quantity of the linoleic tetrabromide. Burr & Burr tested the material thus prepared on a group of 3 rats, each receiving 3 drops of methyl linolenate daily, and on a group of 2 rats, each receiving 3 drops of methyl linoleate daily. The average total weight increases of the two groups in 63 days were almost identical, being respectively 31and 31·5 g.

Chemical preparation. In the present experiments two preparations of linolenic hexabromide, derived from linseed oil and melting at 180–182° and at 182·5° respectively, were used for the preparation of methyl linolenate; the Br contents were 62·0 and 63·06 % for the two samples, the theoretical value for linolenic hexabromide being 63·3 %. The iodine values of the two specimens of methyl linolenate used in the feeding tests were 230·6 and 245 respectively.

The linoleic acid prepared from maize oil was converted into the linoleic tetrabromide, the M.P. of which was 115°; the Br contents were 54·4 and 53·7 %, the theoretical value being 53·3 %. This material was debrominated and esterified, and the methyl linoleate thus prepared gave iodine values of 165·6 and 166·5. To avoid oxidation the unsaturated esters were sealed in small tubes filled with nitrogen and kept at 0°. When a tube was in use for feeding the I.V. was tested at intervals to see that no marked oxidation had taken place. Under these conditions the I.V. of the methyl linoleate had fallen after 24 days from 166·5 to 163·1 and that of the methyl linolenate from 245 to 240.

A sample of methyl β-linoleate was prepared by debrominating the liquid form of linoleic tetrabromide. It had an iodine value of 135·2.

The sample of linseed oil used for comparison in the feeding tests had I.V. 180 and contained about 26 % linoleic and about 44 % linolenic acid.

Biological tests. The biological tests were essentially a comparison between the potencies of methyl linoleate and methyl linolenate. Groups of 7–9 rats received a daily dose of 6 drops (0·08 g.) of one of these two substances or served as negative controls with no addition (see Tables I*a* and *b*). The average total weight increase in 35 days for the group receiving methyl linoleate was 23 g., for that receiving methyl linolenate 8 g. and for the negative control group 2 g. As regards the skin lesions of the ankles, all but one of the rats receiving methyl linoleate showed complete healing in 35 days; of those receiving methyl linolenate all showed partial, but none complete, healing in the period, and the negative control group showed no healing at all.

In addition two animals received daily 5 drops (about 0·08 g.) of linseed oil, one received 6 drops (0·08 g.) of methyl β-linoleate and four received 1 drop (0·013 g.) of methyl linoleate. The numbers of rats for these tests are small but reference to Tables I*a* and I*b* shows that the average behaviour of these groups both as regards cure of lesions and resumption of weight increase was intermediate between that of the group receiving 0·08 g. methyl linoleate, and that receiving 0·08 g. methyl linolenate. The average weight increase of 14 g. shown by the rats receiving one drop of methyl linoleate was greater than that of 8 g. shown by the rats receiving 6 drops of methyl linolenate.

The behaviour of one rat was of particular interest. After having received 6 drops methyl linolenate daily for 13 days it had lost 19 g. in weight and was in

Table I*a*. *Total wt. increase in 35 days of young rats which had received a diet devoid of fat for about 120 days, and then were given methyl linoleate, methyl β-linoleate, methyl linolenate, no supplement or linseed oil*

(Results marked with the same symbol *, † etc., apply to the same individual rat in two different tests.)

Litter no.	Sex	Methyl linoleate 0·013 g. daily (g.)	Methyl linoleate 0·08 g. daily (g.)	Methyl β-linoleate 0·08 g. daily (g.)	Methyl linolenate 0·08 g. daily (g.)	No supplement negative controls (g.)	Linseed oil 0·08 g. daily (g.)
4330	♂	17*	13	.	.	−10*	.
	♀	.	.	.	14†	− 1	.
	♀	16	.	.	7†	.	.
4329	♂	.	19	.	2	−17	20
	♀	.	30	.	9	2‡	20
	♀	12‡	.	.	.	.	.
4338	♀	.	31	16	8	5§	.
	♀	.	18	.	−19‖ (13 days)	.	.
	♀	13§	39‖	.	25	.	.
4350	♂	.	.	.	3	3	.
	♀	.	10	.	3	5	.
Av.		14	23	16	8	2	20

Table I*b*. *Healing of skin symptoms in 35 days. Same series as above. Three degrees of healing recognized: complete* ++, *partial* +, *none* θ

Litter no.	Sex	Methyl linoleate 0·013 g. daily	Methyl linoleate 0·08 g. daily	Methyl β-linoleate 0·08 g. daily	Methyl linolenate 0·08 g. daily	No supplement negative controls	Linseed oil 0·08 g. daily
4330	♂	++*	++	.	.	θ*	.
	♀	.	.	.	+†	θ	.
	♀	+	.	.	+†	.	.
4329	♂	.	++	.	+	θ	+
	♀	.	++	.	+‡	θ	+
	♀	+‡	.	.	.	.	.
4338	♀	.	++	+	+	θ§	.
	♀	.	++	.	θ‖ (13 days)	.	.
	♀	+§	+‖	.	+	.	.
4350	♂	.	.	.	+	θ	.
	♀	.	++	.	+	θ	.

a very precarious condition. The dose was changed to 6 drops daily of methyl linoleate; the rat at once ceased to lose weight and quickly began to gain, making a total weight increase of 39 g. in the 35 days immediately ensuing (see Table I*a*, *b*, rat 4338 ♀‖). The skin lesions of the ankles were not, however, completely cured within this period. Rats did not usually lose weight when receiving methyl linolenate, so that this is the only case in which so marked a curative action of methyl linoleate in contrast with methyl linolenate was demonstrated.

A certain number of the rats continued to receive the unsupplemented deficient diet after the end of the 35-day period of dosage with methyl linoleate or linolenate, and they were observed for irregular periods varying from 30 to 80 days. Their behaviour showed a powerful and sustained action of methyl

linoleate and a weak and transitory one of methyl linolenate. Of 5 rats receiving the former, all continued to gain in weight slowly and the unhealed lesions of skin and tail, excluding the ankles which were already healed, progressed steadily towards cure. Of 3 rats which received 6 drops of methyl linolenate, only one made any sustained weight increase and none showed any progress of the cure; the ankles, which were only partly healed at the end of the period of dosage, never showed any tendency to complete healing (see Table II).

Table II. *Subsequent behaviour of rats receiving the unsupplemented fat-free diet in the period immediately following a 35-day period of dosage with 0·08 g. daily of methyl linoleate or methyl linolenate*

Litter no.	Sex	Previous daily supplement	Length of period without supplement days	Total wt. increase in period without supplement g.	Behaviour of symptoms in period without supplement
4330	♂	Methyl linoleate 0·08 g.	80	33	All skin and tail lesions continuing to progress towards normal
4329	♂	,,	70	28	
4329	♀	,,	70	20	
4338	♀	,,	30	16	
4338	♀	,,	36	13	
4330	♀	Methyl linolenate 0·08 g.	45	0 (+7, −7)	No progress in healing of lesions. Partly healed ankles showing no improvement
4329	♀	,,	42	1 (+4, −3)	
4329	♂	,,	45	12	

All the experimental evidence thus obtained, therefore, went to indicate that methyl linoleate exercised a potent and prolonged action in counteracting the effects of a fat-free diet, while methyl linolenate had only a weak and transitory action.

Discussion. The difference thus established is of especial interest since there is general agreement that whilst linoleic acid is normally a constituent of the body lipins, linolenic acid is only present in small amounts, as the result of administering it in exceptionally large amounts [Ellis & Isbell, 1926; Spadola & Ellis, 1936]. Thus Hartley [1909] found evidence of the presence in the liver, heart and kidney of pigs of unsaturated acids with one, two, and four double bonds. Snider & Bloor [1932–3] found oleic, linoleic and arachidonic acids in ox liver lecithin, and failed to find linolenic acid, a result confirmed by Klenk & Schoenebeck [1932]. Turner [1930] found no linolenic acid in sheep's liver. Eckstein [1929] found linoleic and arachidonic acids in rat tissues but no evidence of linolenic acid. Levene & Rolf [1926] described linolenic acid as exceeding linoleic acid in amount in liver lecithin, but the hexabromide described, being soluble in ether, does not agree in properties with linolenic hexabromide. It seems certain, therefore, that though linolenic acid may be consumed in large quantities it is not taken up as a constituent of the body lecithins. Since it is not stored in the body it is not surprising that after its administration has been stopped, no further increase in weight or improvement of symptoms takes place in the rat. Linolenic acid must be more rapidly transformed on entering the body than in linoleic acid, and it is possibly owing to this change that methyl linolenate is so markedly inferior to methyl linoleate in its effect in counteracting the effects of a fat-free diet.

Effects of elaidized linoleic and linolenic acids. Tange [1932] added 0·5% elaidic acid to the fat-free diet of 5 rats of which two failed to show weight increase.

He reports that this acid was ineffective in curing symptoms and behaved like oleic acid.

Preparation. Linoleic and linolenic acids were elaidized according to the directions of Griffiths & Hilditch [1932] and the methyl esters were used for the biological test. The esters were orange in colour; the I.V. of the original methyl α-linoleate was 165·6, and after elaidization it was 157·2. The I.V. of the original methyl α-linolenate was 230·6, and after elaidization it was 215.

Biological test. One rat received 0·08 g. daily of elaidized methyl α-linoleate and showed a weight increase of 24 g. in the experimental period, with partial healing of skin lesions. Another rat received the same amount of elaidized methyl α-linolenate and showed no weight increase but about the same degree of healing; it was found to be suffering from the chronic lung affection of rats which would affect adversely its capacity to put on weight (see Table III). The elaidized methyl α-linoleate still contained a large proportion of non-elaidized linoleate; the degree of healing was inferior to that previously observed with the same dose of methyl linoleate.

(2) *Examination of the potencies of certain oxidation products of linoleic and linolenic acids.* (*a*) *Tetrahydroxystearic acids: tests on mixtures of α- and β-, and of γ- and δ-tetrahydroxystearic acids.* (*b*) *Dioxidostearic acid.* (*c*) *Hexahydroxystearic acids: linusic and isolinusic acids.*

(*a*) *Tetrahydroxystearic acids.* Nothing is known as to the changes which linoleic and linolenic acids undergo in the body. It seemed possible that, as a further stage in metabolism, oxidation products might be formed. This possibility cannot be tested exhaustively even for the hydroxystearic acids, since few of them have been isolated. Thus eight racemic forms of tetrahydroxystearic acid may theoretically be derived from linoleic acid but only four of these are known; α- and β-tetrahydroxystearic acids, melting respectively at 154·7° and 171·3°, have been obtained by the action of $KMnO_4$ on linoleic acid in alkaline solution. The γ- and δ-forms were obtained by Nicolet & Cox [1922] by the addition of HOBr to linoleic acid and the conversion of the hydroxybromo-derivatives into the tetrahydroxy-acids. The γ-form melted at 144·5°, the δ-form at 135°.

Preparation. Mixtures of the 9:10:12:13-α- and β-tetrahydroxy-acids were prepared with M.P. 164–166°, and of the 9:10:12:13-γ- and δ-tetrahydroxy-acids with M.P. 145–146° by the above methods. These two mixtures were used for biological tests.

Biological tests. Three rats received 0·2 g. daily of the mixture of the α- and β-forms, and one rat received 0·2 g. of the mixture of γ- and δ-forms (see Table III), but no weight increase or amelioration of skin symptoms occurred in any case. Thus no indication of the activity possessed by linoleic acid was shown by these tetrahydroxy-addition products of it.

(*b*) *Dioxidostearic acid. Preparation.* Dioxidostearic acid was prepared by the method of Green & Hilditch [1935], by the oxidation of methyl linoleate with perbenzoic acid in chloroform solution and recrystallization from hot 70% alcohol. It melted at 78–79°.

Biological test. A daily dose of 0·2 g. of the preparation was added to the diet of one rat for 35 days. It showed no gain in weight and no healing of skin symptoms; a litter-mate negative control gained 7 g. in the same period (see Table III).

Table III. *Total wt. increase and healing of skin symptoms in young rats which had received a diet devoid of fat for about 120 days and then various supplementary materials for differing periods*

Litter no.	Sex	Material	Dose daily g.	Duration of dosing days	Wt. increase g.	Healing of symptoms
4329	♂	Elaidized methyl α-linoleate	0·08	35	24	+
4329	♀	Elaidized methyl α-linolenate	0·08	35	0 (pneumonia)	+
4075	♀	9:10:12:13-α- and β-tetrahydroxy-stearic acid	0·2	35	0	θ
4076	♀	,, ,,	0·2	35	0	θ
4330	♂♀	,, ,,	0·2	35	− 9	θ
4330	♂	9:10:12:13-γ- and δ-tetrahydroxy-stearic acid	0·2	35	− 9	θ
4338	♀	Dioxidostearic acid	0·2	35	− 3	θ
4350	♂♀	Methyl docosahexaenoate	0·06–0·1	35	24	? < +
4350		,,	0·06–0·1	35	21	? < +
4273	♂	Chaulmoogra oil	0·16	13	− 5	θ
		Chaulmoogric acid	0·2	5	0	θ
4330	♂	Methyl arachidate	0·3	15	− 9	θ
4273	♀♂	9:10:12-trihydroxystearic acid	0·2	35	− 4	θ

(*c*) *Hexahydroxystearic acids*: *linusic and* iso*linusic acids*. Thirty-two racemic isomerides of the hexahydroxystearic acids derived from linolenic acid are theoretically possible; the racemic linusic and *iso*linusic acids obtained by oxidation with $KMnO_4$ of the unsaturated acids of linseed oil [Rollett, 1909] are the only ones known.

Preparation. Linusic acid, M.P. 196–197° and *iso*linusic acid, M.P. 172–174°, were prepared as above.

Biological tests. The results are set out in Tables IV *a*, *b*. Five rats received 0·2 g. daily of linusic acid; 7 received the same amount of *iso*linusic acid, and 2 received 0·1 to 0·13 g. daily of a mixture of both acids. All tended to show some weight increase, the 12 animals which received 0·2 g. daily giving an average total weight increase for the experimental period of 6 g., as compared with an average total weight increase of 3 g. for 7 litter-mate negative controls with no supplement, and of 20 g. for 5 litter-mate positive controls receiving 3–10 drops (0·05–0·16 g.) linseed oil daily. This performance appears to represent a definite though small weight increase by the rats receiving the hexahydroxystearic acids, not greatly inferior to that of rats receiving methyl linolenate, as already described (see Table I *a*). As regards the healing of skin lesions the rats receiving the hexahydroxystearic acids behaved like the negative controls. It is regrettable that rats receiving methyl linolenate were not included simultaneously in this comparison; when it was begun the inferiority of linolenic to linoleic acid had not been established and it was thought that the mixture of both these acids, represented by linseed oil, would be a satisfactory material for a positive control.

The faeces of the rats fed with the tetrahydroxy-acids in the previous experiment and the hexahydroxy-acids in the present experiment were large and pale in colour and obviously contained a large amount of the hydroxy-acids administered. Indeed it is doubtful how much of substances of such high melting points and so insoluble in water could be absorbed; the amounts absorbed can only have been a small fraction of the dose administered. The solubility in water of the hexahydroxy-acids is appreciably greater than that of the tetrahydroxy-acids, and possibly more of these may pass through the intestinal wall. For a part of the experimental period one rat received ethyl linusate and another ethyl

Table IV*a*. *Total wt. increase in 35 days of young rats which had received a diet devoid of fat for about 120 days, and then were given linusic acid,* iso*linusic acid, no supplement or linseed oil*

Litter no.	Sex	Linusic acid 0·2 g. daily g.	*iso*Linusic acid 0·2 g. daily g.	No supplement g.	Linseed oil 3–10 drops daily (0·05 to 0·16 g.) g.
4075	♀	9	9	.	17
	♂	.	.	8	.
4076	♀	11	12	4	17
4273	♀	− 4*	− 1*	− 1	.
	♀	0*	.	.	.
	♂	.	−13	−13	25
4330	♀	.	4	− 1	.
4350	♀	6	8	8	10
3262	♀	.	7	4	25
		Linusic and *iso*linusic acids mixed 0·1–0·13 g.			
2907	♀	9		− 1	.
2931	♂	35†		6†	26†
Av.		5	6	3	20
		8			

* Experiment lasted 30 instead of 35 days.
† Experiment started on 75th instead of about 120th day.

Table IV*b*. *Healing of skin symptoms in 35 days. Same series as in Table IVa. Three degrees of healing recognized, complete ++, partial +, and none θ*

Litter no.	Sex	Linusic acid 0·2 g. daily	*iso*Linusic acid 0·2 g. daily	No supplement	Linseed oil 3–10 drops daily (0·05–0·16 g.)
4075	♀	θ	θ	.	++
	♂	.	.	θ	.
4076	♀	θ	θ	θ	+
4273	♀	θ*	θ*	θ	.
	♀	θ*	.	.	.
	♂	.	θ	θ	+
4330	♀	.	θ	θ	.
4350	♀	θ	θ	θ	++
3262	♀	.	θ	θ	+
		Linusic and *iso*linusic acids mixed 0·1–0·13 g.			
2907	♀	θ		θ	.
2931	♂	θ†		θ†	++†

* Experiment lasted 30 instead of 35 days.
† Experiment started on 75th instead of about 120th day.

*iso*linusate, instead of the corresponding acids, in the hope that the esters might be better absorbed, but weight increase was not favourably affected. An attempt to promote absorption was also made by adding about 0·25 g. daily of coconut oil simultaneously with the dose of 0·2 g. daily of linusic acid to the diet of one rat for a part of the experimental period, but without result.

The experiments with these hydroxy-derivatives cannot, therefore, be regarded as conclusive since there was no test of the amount absorbed. The small positive effect on weight increase observed in the case of linusic and *iso*linusic acid seems, however, even more convincing when it is remembered that a considerable proportion of the original dose was certainly excreted in the faeces.

(3) *Comparison of the potency of the unsaturated acids from lard and from linseed oil*

When Burr & Burr [1929] first described the deficiency disease produced by a fat-free diet they found that the condition was cured or prevented by the inclusion of 2 % lard in the diet. They referred to lard as one of the best curative fats and suggested that this may possibly be due to the presence of arachidonic acid in the lard fat. Later Burr *et al.* [1932] tested the efficacy of arachidonic acid by replacing 10 % of a mixture of the esters of linoleic and linolenic acids by methyl arachidonate and comparing the effect of 3 drops of the mixture with that of 3 drops of the original ester mixture. The mixture containing the arachidonate proved to be appreciably less active in promoting weight increase than the original mixture. Turpeinen [1938], on the other hand, estimated the effect on weight increase of pure methyl arachidonate and found it three times as potent as methyl linoleate. He does not, however, give any account of its healing effect on the skin lesions, though he found it potent to restore the oestrous cycle towards normal.

The amount of arachidonic acid in lard is usually less than 0·1 % [cf. Ellis & Isbell, 1926; Spadola & Ellis, 1936]; the sample used by Burr & Burr contained 0·06 % so that it does not seem probable that the potency of the lard depended to any great extent on its content of arachidonic acid. Its effect must, therefore, have been due chiefly to its content of linoleic acid. In the lard derived from maize-fed pigs used by Burr & Burr, the percentage of linoleic acid was 6·7. The percentage of linoleic acid in lard is extremely variable, being dependent on the previous diet of the pig. The lard of an animal fed with a ration containing oils rich in linoleic acid may contain as much as 20 % of linoleic acid, and when soya bean has been included in the food 35 to 36 % has been found [Banks & Hilditch, 1932; Ellis & Isbell, 1926; Ellis *et al.* 1931], but usually the linoleic acid content is below 10 %. Percentages varying from 23 to 70 are given for the linoleic, and from 18 to 49 for the linolenic, acid content of linseed oil, but since the proportions of the two acids vary inversely to one another, the iodine value of the oil is fairly constant. Burr & Burr [1929] and Burr *et al.* [1932] found lard less potent than linseed oil.

Preparation. The methyl esters of the unsaturated acid fraction were prepared from American lard, distilled *in vacuo*, and a fraction with I.V. 111·7 was used for the biological tests. This would correspond with a mixture of about 70 % oleic and about 30 % linoleic acids.

A fraction of the unsaturated acids from linseed oil was also prepared; it had I.V. 199 to 200 and contained approximately 20 % oleic, 30 % linoleic and 50 % linolenic acid. The linoleic acid content of the fraction derived from the lard was thus about the same as that of the fraction derived from the linseed oil, but the linseed oil fraction contained in addition nearly double the amount of linolenic acid.

Biological tests. Groups of 4 litter-mate rats received 3 to 5 drops (about 0·05–0·08 g.) daily of the fraction of unsaturated acids from lard and from linseed oils, two animals in each group receiving 3 and two 5 drops. The average

total weight increase was 29 g. in both groups, and all the animals showed partial healing of the ankles to about the same extent (see Table V). It is therefore concluded that the activities of these two fractions were about the same.

Table V. *Total wt. increase and healing of skin symptoms in 35 days of young rats which had received a diet devoid of fat for about 120 days, and were then given unsaturated fatty acid fractions from lard or linseed oil*

Litter no.	Sex	Linseed oil acids 3–5 drops (0·05–0·08 g.) g.	Lard acids 3–5 drops (0·05–0·08 g.) g.	Linseed oil acids 3–5 drops (0·05–0·08 g.)	Lard acids 3–5 drops (0·05–0·08 g.)
3262	♂	36	27	+	+
	♀	21	21	+	+
1255's	♂	40	48	+	+
1258's	♀	16	22	+	+
Av.		28	29	.	.

These experiments seem to afford additional confirmation of the great superiority of linoleic over linolenic acid as a curative agent, for the two fractions contained about the same amount of linoleic acid, but the linseed oil contained a large amount of linolenic acid in addition.

Lard and linseed oil vary so much in their linoleic acid contents that considerable discrepancies between the results of different workers must be expected, and feeding experiments in which different samples of lard are used can be justly compared only when the linoleic acid contents have first been determined.

(4) *Comparison of the potencies of raisin seed oil and of linseed oil*

Raisin seed oil, which has only comparatively recently appeared on the market, is a by-product of the wine industry. A sample of material of Californian origin was contributed by Messrs W. J. Bush and Co., Ltd. for a biological test. This sample of raisin seed oil proved to have I.V. 122·4. It contained 93·5 % of fatty acids, the I.V. of which was 126·7, with 1·07 % of unsaponifiable matter. On bromination no ether insoluble bromide was obtained, and the percentage of linoleic acid was therefore calculated to be 38·6, no evidence of the presence of linolenic acid having been obtained.

The linseed oil used was that already described (p. 2166) as having a content of about 26 % linoleic, and about 44 % linolenic, acid.

Biological test. Four litter-mate rats received for 35 days 5 drops (about 0·08 g.) daily of the raisin seed oil, four received the same amount of linseed oil, and

Table VI. *Total wt. increase and healing of skin symptoms in 35 days of young rats which had received a diet devoid of fat for about 120 days, and then were given linseed oil or raisin seed oil or no supplement*

Litter no.	Sex	Linseed oil 5 drops (0·08 g.) g.	Raisin seed oil 5 drops (0·08 g.) g.	No supplement negative controls g.	Linseed oil 5 drops (0·08 g.)	Raisin seed oil 5 drops (0·08 g.)	No supplement negative controls
4075	♂	39	52	10	+	+ +	θ
	♂	.	31	.	.	+ +	.
	♀	16	18	.	+ +	+ +	.
4076	♂	28	.	0	+	.	θ
	♀	17	12	5	+	+ +	θ
Av.		25	28	5			

three received no supplement. The average total weight increases in the period were 28, 25 and 5 g., respectively. All the four rats receiving raisin seed oil, and one rat receiving linseed oil, showed complete healing of the ankles in the period; the other three rats receiving linseed oil showed partial healing; the three receiving no supplement showed no healing (see Table VI).

A slight superiority of raisin seed oil over linseed oil seems therefore to be established. The result harmonizes well with the other results obtained and confirms the marked inferiority of linolenic to linoleic acid, since the linseed oil sample had a large content of linolenic acid, but somewhat less linoleic acid than the raisin seed oil.

(5) *Test of the potency of the methyl ester of the docosahexaenoic acid isolated from cod liver oil by Farmer & Van den Heuvel*

There is a mass of evidence in the literature showing that cod liver oil, although ineffective in curing the skin lesions produced by a fat-free diet, is effective in promoting weight increase. Thus Sinclair [1929–30] found that this oil, added as 10 % of the otherwise fat-free diet of rats, did not cure scaliness, whereas 1 % of lard was effective. Graham & Griffith [1930–31] reported a daily dose of 9–12 drops of cod liver oil as ineffective while wheat germ oil and lard cured well. Burr *et al.* [1930–31] gave 2–5 drops of cod liver oil daily and found weight increase renewed though the scaly tail was not cured. McAmis *et al.* [1929] found the rate of weight increase of fat-deprived rats much improved when 0·02 g. cod liver oil was added to the daily ration. These results would indicate the absence of linoleic acid from cod liver oil and the presence of other unsaturated acids able to promote weight increase but unable to alleviate the skin lesions [Burr *et al.*, 1930–31]. From the figures given by Banks *et al.* [1933] for the iodine values of the unsaturated acids derived from the depot fat of rats which had received a fat-free diet supplemented with cod liver oil, it is clear that when cod liver oil was given, highly unsaturated acids were laid down in the depot fat.

The composition of cod liver oil was investigated by Guha *et al.* [1930] who examined the methyl ester fractions of the unsaturated acids obtained by distillation under a pressure of 1 mm. If it is assumed that the iodine value of the fraction containing 18 carbon atoms is entirely due to the presence of oleic and linoleic acids, the proportion of linoleic acid would correspond with a content of about 12 % in the original oil. The fraction containing 18 carbon atoms formed 18–32 % of the total acids and absorbed from 2·8 to 4 atomic proportions of hydrogen. There was, however, no actual identification of linoleic or linolenic acid, and the same values could be obtained from mixtures of isomeric acids. In view of the absence of healing of the skin lesions when large doses of cod liver oil are added to the fat-free diet, it is difficult to believe that any appreciable amount of linoleic acid can be present in the oil [cf. Burr *et al.*, 1930–31].

Farmer & Van den Heuvel [1938, 2] have recently pointed out that, in all fractionations of the methyl esters of cod liver oil previously carried out, changes occurred which pointed to the occurrence of alterations in structure during distillation. After distilling the esters, even at a pressure of 0·1 mm., evidence of the presence of conjugated double bonds was obtained whereas there was no evidence of their presence before distillation. Much polymerization also occurred and the fractions isolated consisted of constant boiling mixtures. Farmer & Van den Heuvel [1938, 1] have, therefore, used the method of molecular distillation in which pressure was reduced to 0·001 mm. In this way, from 950 g. unsaturated acids, 225 g. of an acid containing 22 carbon atoms and 6 double bonds was isolated, no conjugated double bonds being present in the molecule

[Farmer & Van den Heuvel, 1938, 2]. The fraction containing 18 carbon atoms, when hydrogenated, gave pure stearic acid; the value for the hydrogenation number indicated that it contained 2·7 double bonds, so that in composition it approximated more nearly to linolenic than to linoleic acid.

A sample of the methyl ester of the C_{22} hexaenoic acid was contributed by Dr Farmer and was used for a biological test.

Biological test. The methyl ester of the docosahexaenoic acid had a strong fishy odour and was much disliked by the rats. It also showed in a high degree the property already mentioned (see p. 2164) of destroying the upper layers of the skin about the mouth, wherever it was allowed to come into contact with them, though no damage appeared to be caused to the mucous membrane of the mouth or intestinal tract. By intimate admixture with the daily yeast dose its consumption was satisfactorily secured.

Two rats received a daily dose of 5–8 drops (0·06–0·1 g.) of the material for 35 days, in which period the weight increase was 21 and 24 g. Such weight increase much improved the appearance of the animals, making it difficult to decide whether there was or was not some small improvement in the skin lesions; the ankles were, however, still rough and quite unhealed at the end of the period (see Table III). After the 5 weeks' experimental period the two animals were maintained on the same diet, without addition of the daily dose of acid, for another 14 days, on the chance that cure of the skin symptoms might continue and become more convincing as had been found to be the case with linoleic acid, but this did not occur. One rat, however, added another 5 g. of weight and the other 12 g. in this period. The behaviour of the rats was therefore in marked contrast with that of rats receiving linoleic acid, and there seem to be adequate grounds for distinguishing between the effects of the highly unsaturated acids of cod liver oil, which enable body fat to be laid down, and those of linoleic acid, which in addition to promoting weight increase, produce rapid amelioration and final cure of skin lesions.

(6) *Tests for potencies of chaulmoogra oil and chaulmoogric acid, methyl arachidate and 9:10:12-trihydroxystearic acid*

Chaulmoogra oil. The sample of oil used had I.V. 101·3. One rat received 10 drops (about 0·16 g.) daily for 13 days, but it lost weight and the oil appeared to be actually toxic (see Table III).

Chaulmoogric acid was prepared from chaulmoogra oil. It had M.P. 65·5° and I.V. 91·1. The same rat as above received 0·2 g. daily for 5 days; the material was badly consumed but appeared quite inactive, a result in agreement with that of Turpeinen [1938].

Methyl arachidate. In view of Turpeinen's work on the activity of arachidonic acid, it was of interest to see whether any evidence of activity could be obtained which would indicate the unsaturation within the body of arachidic to arachidonic acid. From arachis oil a fraction of saturated acid M.P. 72–75° was prepared, consisting mainly of arachidic acid. This was converted into the methyl ester. Owing to the high melting point the material would probably be badly absorbed in the biological test.

Biological test. One rat received 0·3 g. daily of the material for 15 days during which period it lost 9 g. and showed no alleviation of symptoms. The result was regarded as completely negative (see Table III).

9:10:12-Trihydroxystearic acid. The acid was prepared by oxidizing ricinoleic acid with $KMnO_4$; crystallized from alcohol it melted at 131–134°.

Biological test. One rat received 0·2 g. daily of the material for 35 days; it lost 4 g. and showed no improvement in skin symptoms. Like the ricinoleic acid tested by Turpeinen, it was thus completely inactive (see Table III).

Summary

1. The principal symptoms of the deficiency disease described by Burr & Burr as affecting rats maintained on a complete diet devoid only of fat were reproduced.

2. The efficacy of various materials adequately to supplement the fat-free diet was studied. Resumption of weight increase and healing of skin symptoms were the criteria used.

3. A rough method of estimating quantitatively the rate of healing of skin lesions is described. The importance of using some additional criterion more specific than weight increase is stressed.

4. The efficacies of methyl linoleate and methyl linolenate were compared; methyl linoleate was found much more potent and lasting in action than methyl linolenate, which possessed perhaps no more than one-sixth of the potency of methyl linoleate.

5. Various oxidation products of linoleic and linolenic acid were prepared and tested. Mixtures of α- and β- and of γ- and δ-tetrahydroxystearic acids in daily doses of 0·2 g. were completely ineffective in promoting weight increase or curing skin lesions in rats. Dioxidostearic acid was similarly inactive.

The hexahydroxy-derivatives, linusic and *iso*linusic acids, given in daily doses of 0·2 g., failed to benefit skin lesions but promoted a small but definite weight increase nearly equal to that evoked by administration of methyl linolenate in a daily dose of 0·08 g. All these oxidation products were solid substances of high melting point and were certainly absorbed only to a very limited extent by the rat's intestine.

6. The potency of a fraction of the unsaturated acids from lard was compared with that of a similar fraction from linseed oil and was found to be about the same, both as regards promotion of weight increase and healing of skin lesions.

7. A similar comparison was made of linseed oil with raisin seed oil; the latter was found slightly the more potent by both criteria. The linoleic acid contents of these two materials and of the fractions of linseed oil and lard were not greatly dissimilar but the linseed oil and its acids both contained 40 % to 50 % linolenic acid in addition. These results therefore confirm the superior potency of linoleic to linolenic acid.

8. The methyl ester of the docosahexaenoic acid isolated by Farmer and Van den Heuvel from cod liver oil was tested and found to be potent in promoting weight increase but to have little or no action in curing skin lesions.

9. Chaulmoogra oil and chaulmoogric acid, methyl arachidate and 9:10:12-trihydroxystearic acid were also tested and found inactive.

10. It is concluded that the ability of unsaturated fatty acids to supplement a fat-free diet in promoting weight increase is not necessarily associated with ability to heal skin lesions.

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CCLXXXII. THE NATURE OF THE FATTY ACIDS STORED BY THE LIVER IN THE FAT-DEFICIENCY DISEASE OF RATS

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The part played by those unsaturated fatty acids which have been shown to be essential for the maintenance of rats in normal health is as yet quite unknown. A first step in elucidating this problem was to gain further knowledge as to the nature of the fatty acids stored when the animals were kept on a fat-deficient diet, and to observe the changes which occurred in them when the curative acids were fed.

At the conclusion of feeding experiments carried out in co-operation with Hume *et al.* [1938], we selected five groups of rats and examined the fatty acids present in the livers. The long period of preparation necessary before symptoms are sufficiently well established for curative measures to be tested made strict economy desirable and in most cases before the rats were killed the negative controls had been utilized for further tests. It was not, therefore, possible to obtain a large group of negative controls for the isolation of the liver fatty acids and we had not sufficient material to make a separation into phospholipin and neutral fat. In normal animals the greater part of the liver fatty acids comes from the phospholipins: in the ox, Bloor & Snider [1930] give the proportion of phospholipin fatty acid to acid from neutral fat as 6 : 1; this figure is in close agreement with those given by Klenk & Schoenebeck [1932]. Theis [1928], on the other hand, gives the ratio as 5 : 4. Monaghan [1932] found that in fasting animals the proportion of neutral fat was much raised. It seems probable that most of the highly unsaturated fatty acids came from the phospholipin fraction, but definite evidence on this point is still required.

The five groups of rats selected were:

(1) *Two rats* (♂+♀) *which served as our negative controls.* These weighed 42 and 45 g. respectively when placed on the fat-free diet. The diet was continued for 18 weeks and after the growth curve had remained flat for 6 weeks they received respectively daily doses of 0·2 g. linusic and *iso*linusic acids. These doses were continued for 5 weeks, during which period the weight of the linusic-fed rat increased by 5 g. and that of the one fed with *iso*linusic by 8 g. For the next $7\frac{1}{2}$ weeks before being killed they again received the fat-deficient diet; these were chosen as being the nearest to completely negative controls available at the end of the feeding experiments.

Although the linusic-fed rat only gained 5 g. in weight during the 5 weeks period of dosing, $17\frac{1}{2}$ g. increase was recorded in the $3\frac{1}{2}$ weeks immediately following cessation of the dose, then the weight remained stationary until the rat was killed $3\frac{1}{2}$ weeks later. Since both rats had received the fat-free diet for 7 weeks before they were killed and prior to that the comparatively inactive hexahydroxy-acids had been fed, these rats may probably be regarded as characteristic examples of rats fed on a fat-free diet.

(2) *Rats receiving methyl linoleate.* Three rats (♀) weighing 44, 45 and 46 g. respectively when transferred to the fat-deficient diet, were utilized. One of these, after it had been on this diet for 20 weeks, was given a daily dose of 6 drops methyl linoleate for a period of 6 weeks during which its weight rose from 121 to 152 g. A month's cessation of dosing followed during which the rat's weight increased by 16 g. and finally, for its last 3 weeks, it received 1 drop linoleate daily. The other two rats, after receiving the fat-deficient diet for 6 months, were both dosed with 1 drop linoleate daily for 38 days, the respective gains in weight being 12 and 15 g. This dose was shown to produce amelioration but not cure of symptoms and the rats, therefore, probably do not show the typical results of rats fed with the optimum linoleate dose.

(3) *Rats receiving methyl linolenate.* Two rats (♀) which had received the fat-free diet for 16 weeks were given, respectively, during the course of 1 month, a daily dose of 0·2 g. dioxidostearic acid and 1 drop of methyl linolenate. Finally for 38 days before they were killed, each received 6 drops linolenate daily. The effects produced may therefore be considered as characteristic for rats fed with methyl linolenate.

(4) *Rats receiving linseed oil.* Six rats (3 ♂, 3 ♀) had received the fat-free diet for from 8 to 10 months. After 4 months one had been given 0·2 g. linusic acid daily for 5 weeks and then after a month's interval a daily dose of 0·2 g. trihydroxystearic acid for another period of 5 weeks. In all cases for 2 to 3 months before being killed, each rat had received a supplement of 5 to 15 drops linseed oil daily. The typical fatty acids stored by rats on a diet containing linseed oil might, therefore, be expected.

(5) *Rats fed with docosahexaenoic acid methyl ester (from cod liver oil).* Two rats were utilized (1 ♂, 1 ♀) which had served as negative controls for 6 months. During 2 weeks they each received a daily dose of 8 drops of the above ester [Farmer & Van den Heuvel, 1938] and for the subsequent 3 weeks, 5 drops daily. Dosage had ceased a fortnight before the rats were killed. Possibly if the rats had been killed whilst the diet still contained the ester supplement, the I.V. of the liver fatty acids might have been higher.

As soon as the animals were killed, the livers were removed, weighed and immediately thrown into a mixture of equal parts of alcohol, water and caustic potash heated on a water bath. After it had been extracted with ether, the soap solution was acidified and the fatty acids were extracted by light petroleum.

Table I

Supplement	No. rats	Total wt. livers g.	Av. wt. liver per rat g.	Total wt. fatty acids g.	Wt. acids in 100 g. liver g.	% acids (*a*) unsaturated	% acids (*b*) saturated	I.V. unsaturated acids
Group I. None	2 (♂+♀)	16·5	8·2	0·62	3·7	62·1	37·9	121·2
Group II. Methyl linoleate	3 (♀)	21·8	7·3	0·57	2·6	62·1	37·9	163·6
Group III. Methyl linolenate	2 (♀)	15·0	7·5	0·36	2·4	62·3	37·7	215·0
Group IV. Linseed oil	6 (3♂+3♀)	55·0	9·2	1·25	2·3	61·7	38·3	202·8
Group V. Methyl ester of cod liver oil acid (docosahexaenoic acid)	2 (♂+♀)	17·3	8·7	0·50	2·9	61·6	38·4	162·3

Three interesting facts emerge from the results recorded in Table I:

(1) The weight of fatty acid calculated per 100 g. liver tissue was considerably higher in the negative controls than in the rats which had received a supplement of unsaturated acid.

(2) Whatever the diet, the proportion of the weights of saturated and unsaturated acids remained extraordinarily constant, the ratio being 38 : 62. Sinclair [1935] found that the proportion of saturated to unsaturated acids in the rat liver phospholipin fraction was 33·6 : 60·9.

(3) The iodine value of the unsaturated acids varied greatly, that of the acids from the negative controls being much lower than that from those rats which had received the doses of the esters of the unsaturated acids.

The rats which had been fed with the linolenic ester or with linseed oil contained the most highly unsaturated fats in their livers. This may possibly be explained by the fact that the dose of linoleate fed to Group II of the rats was known to be well below the optimum and that in the case of the rats dosed with the cod liver oil acid, the dose had ceased a fortnight before the rats were killed.

After the iodine value had been determined, each unsaturated fraction was brominated in ether solution at 0°; after standing overnight in the cold room, the solid ether-insoluble bromides were separated, washed well with ether, extracted with benzene, dried to constant weight and the bromine was estimated. The original weight of unsaturated acid present in the ether-insoluble bromides was then calculated. After the ether-soluble bromides had been weighed they were divided into a liquid fraction soluble in light petroleum and a solid residue small in amount and insoluble in light petroleum. The percentage of bromine was then determined in the petroleum-soluble fraction and the corresponding weight of unsaturated acid calculated. These results are set forth in Table II.

Table II

Supplement to fat-deficient diet	Wt. acids brominated g.	Ether-insoluble bromides			Ether-soluble bromides						% of total acids (calculated from % Br in bromides)			
						(*a*) Petroleum-insoluble			(*b*) Petroleum-soluble liquids			Petroleum		
		Wt. g.	M.P. °C.	% Br.	Total wt. g.	Wt. g.	M.P. °C.	% Br	Wt. g.	% Br	Ether insol.	Insol.	Sol.	Total
Group I:		(*a*) Benzene-soluble												
None	0·2576	0·0264	203–4	61·8	0·41	0·04	190–5	—	0·368	39·76	3·8	5·9[1]	86·0	95·7
Group II:		(b) Benzene-insoluble												
Methyl linoleate	0·2459	0·0376	226–30[3]	64·3	0·43	0·07	200–5	—	0·363	44·78	5·3	10·9[1]	84·3	100·5
Group III:														
Methyl linolenate	0·1406	0·0831	Decomp.[3] above 260	69·0	0·26	0·11	145–8 with decomp.	—	0·149	45·94	17·6	28·7[2]	57·0	103·3
Group IV:														
Linseed oil	0·6506	0·2287	Decomp.[3] above 260	—	1·13	0·27	140–5 with decomp.	—	0·859	42·96	—	15·2[2]	57·0	—
Group V:														
Methyl ester of cod liver oil acid	0·2450	0·0908	Decomp.[3] above 260	70·76	0·41	0·14	—	62·3	0·263	39·1	16·1	21·6	67·0	104·7

[1] Calculated on the assumption that the bromides contain 61·8% Br.
[2] Calculated on the assumption that the bromides contain 63·3% Br.
[3] Traces of bromide of similar M.P. were found in the benzene extract.
Theory for decabromodocosanic acid: 70·8% Br.
Theory for octabromodocosanic acid: 65·8% Br.
Theory for octabromoarachidonic acid: 67·7% Br.
Theory for hexabromoarachidonic acid: 61·06% Br.
Theory for hexabromostearic acid: 63·31% Br.

The nature of the unsaturated acids present in the liver

(1) *Group of negative controls.* The solid ether-insoluble bromide prepared from the negative controls was completely soluble in hot benzene and octa- or deca-bromides of acids containing 20 or 22 carbon atoms were therefore absent. The benzene-soluble bromide melting sharply at 203–4° does not seem to have been previously described. Linolenic hexabromide (M.P. 182–4°) was not present.

Analysis agreed with the formula $C_{20}H_{34}O_2Br_6$. (Found: Br, 61·80; C, 29·82; H, 3·97 %. $C_{20}H_{34}O_2Br_6$ requires Br, 61·06; C, 30·54; H, 4·32 %.) The bromide must, therefore, be derived from a C_{20} acid containing three ethylene linkages, possibly a dihydroarachidonic acid, but arachidonic acid itself is absent.

Sinclair [1935] separated the phospholipins from the total fat contained in the carcasses of rats fed on a fat-deficient diet and found that the ether-insoluble bromides contained 62·2 % Br, a number appreciably lower than that required for linolenic hexabromide (63·32 % Br); he suggested that unsaturated acids hitherto unidentified might be present in these controls. Since Sinclair's results were obtained on the phospholipin fraction, it is probable that the new hexabromide may also have been derived from phospholipin acid. The solubility of Sinclair's bromide in benzene was not definitely stated, nor did he not record its M.P., so that it is not possible to compare further these two substances. In our experiments the acid in the benzene-soluble bromide formed only 3·8 % of the total acids. A further 5·9 % of what appeared to be a less pure specimen of the same acid separated from the petroleum-insoluble fraction of the ether-soluble bromides. After recrystallization this melted at 190–195° but there was insufficient for analysis. In all, therefore, probably rather more than 9 % of the total fatty acid consisted of the dihydroarachidonic acid. The ether-soluble fraction contained bromide corresponding with 86 % of the total acids. The Br content was 39·76 %, so that in addition to oleic dibromide (36·19 % Br) some small proportion of liquid bromides of the more unsaturated acids must have been present.

There was no indication of the presence of either linoleic or linolenic acid.

(2) *Group fed with methyl linoleate.* Here the liver fatty acids yielded ether-insoluble bromides containing 64·3 % Br corresponding to a yield of 5·3 % higher unsaturated fatty acids. The bromide was insoluble in benzene; M.P. 226–30° with decomposition. From the ether-soluble bromide fraction a solid residue was obtained insoluble in light petroleum; when recrystallized, it melted at 200–205° and was probably therefore the dihydroarachidonic acid hexabromide isolated from the negative control; there was insufficient for analysis. The bromine percentage (64·3) in the benzene-insoluble bromide was less than that required by arachidonic octabromide (67·7). Since this fraction melted with decomposition at 226° it was possibly a mixture of arachidonic octabromide and dihydroarachidonic hexabromide, though if present the latter might have been expected to have been dissolved by the benzene. A somewhat similar fraction was obtained by Klenk & Schoenebeck [1932] in separating the acids of the phospholipins of ox liver. This melted with decomposition at 230–33° and by debromination and subsequent dehydrogenation was shown to consist of a mixture of C_{18} and C_{20} derivatives. It seems likely that a mixture of arachidonic acid and its dihydro derivative would behave similarly. Klenk and Schoenebeck identified considerable quantities of linoleic acid as the tetrabromide (M.P. 114–15°) but no evidence of this was found in the fat we examined. The separation of a bromide fraction insoluble in benzene and containing a higher % of Br than that in the C_{20} hexabromide indicate the presence of an acid containing four or more double bonds. As has already been pointed out, only minimal doses of linoleate had been fed to the rats in this group

and it is desirable that the fat should be investigated when adequate supplements of this acid were added to the fat-free diet.

The proportion of unsaturated acid derived from the ether-soluble bromide was 84·3 % of the total amount, in this respect closely resembling the state of affairs existing in the fat of the negative controls. The bromine content of the ether-soluble bromide was, however, higher (44·78 %), resembling that in the linolenic-fed rats.

(3) *Group of rats fed with methyl linolenate.* Here the amount of bromide insoluble in both ether and benzene corresponded to 17·6 % of the total acids. The bromide decomposed above 260° and contained 69·0 % Br; this would be in agreement with a mixture of arachidonic octabromide (67·7 % Br) and the decabromide of the C_{22} acid (70·8 % Br), or possibly a mixture of the octa- and deca-bromides of the C_{22} acid was present. The ether-soluble bromide fraction contained 45·94 % Br and corresponded to a mixture of oleic dibromide with liquid bromides of more unsaturated acids, the corresponding acids forming 57 % of the total. The petroleum-insoluble fraction of the ether-soluble bromides gave an indefinite M.P. at 145–8°; the bromide was not estimated but the corresponding acids probably formed more than 20 % of the whole amount present. Since linolenic hexabromide melts at 182–4°, the presence of an impure specimen of this is not excluded. Great caution has to be exercised in drawing deductions as to the nature of a bromide from its solubility when a large proportion of liquid bromides is also present in the solution.

(4) *Group fed with linseed oil.* Like that from the linolenic-fed rats, the benzene-insoluble bromide decomposed above 260° but unfortunately this fraction was accidentally lost before its weight and its Br content had been determined. The ether-soluble fraction contained 42·96 % Br and the corresponding acid formed 57 % of the total. There was also a fraction insoluble in light petroleum melting at 140–5°, the corresponding acid forming from 10 to 20 % of the whole. These results follow closely those obtained from the rats fed with linolenic acid.

(5) *Group fed with the methyl ester of the cod liver oil acid.* The benzene-insoluble bromide decomposed above 260°; analysis agreed with the formula for the bromide of docosapentaenoic acid $C_{22}H_{34}O_2Br_{10}$. (Found: C, 22·57; H, 3·01; Br, 70·8 %. Theory: C, 23·36; H, 2·72; Br, 70·8 %.) The free acid represented 16 % of the total fatty acids. The ether-soluble fraction also consisted of oleic dibromide with a small proportion of the bromides of the higher unsaturated acids, the latter forming 67 % of the total acids. The petroleum-insoluble fraction was a sticky solid containing 62·30 % Br; the debrominated acid therefore formed 21·6 % of the total.

Discussion

The absence of any acid containing 20 or 22 carbon atoms and four or more ethylene linkages in the rats fed on the completely fat-free diet suggests that unless linoleic or linolenic acid is given, the rat-liver is unable to synthesize the C_{20} or C_{22} acid containing four or more double bonds, essential for the continued existence of the animal. The source of the dihydroarachidonic acid isolated from the livers of the negative controls is not certain. It has been established, largely owing to the work of Sinclair, that the replacement of these highly unsaturated acids takes place extremely slowly, and this is corroborated by the long time that is necessary to establish the symptoms of the fat deficiency disease in rats which had been placed on the fat-free diet immediately after thay had been weaned. The dihydroarachidonic acid was, therefore, probably derived from arachidonic

acid originally present in the rat, its synthesis from the oleic or saturated acids which the rat may still be able to form from carbohydrate is not excluded.

It was unfortunate that the only linoleate-fed rats available were those which had been kept for a very long time on the fat-free diet and then fed for 5 weeks with quite inadequate amounts of linoleate, so that they could not be expected to show typical results. Small as were the doses of linoleate, they resulted in the deposition in the liver of an acid containing more than three double bonds. From the bromine content of this bromide fraction, a mixture of arachidonic and dihydroarachidonic acids may have been present. Possibly because of the insufficiency of the doses of linoleate which had been given, the C_{20} hexabromide, which had been isolated from the negative controls, appeared to be present in the petroleum-insoluble fraction; presumably its bromide had been carried into solution by the large proportion of ether-soluble liquid bromides present.

There is a good deal of evidence in the literature that arachidonic acid is formed when oils containing linoleic acid are fed [Ellis & Isbell, 1926; Eckstein, 1929; Spadola & Ellis, 1936]. There is not, however, always a clear differentiation made between the octabromide of arachidonic acid and the decabromide of the C_{22} acid.

Again from the literature [Ellis & Isbell, 1926; Klenk, 1932; Snider & Bloor, 1933] convincing evidence is present showing that linolenic acid is not stored as such in the body. Our experiments seem to point to its conversion into the C_{22} acid with five double bonds. The function of the liver here is not to desaturate the 18-carbon chain but, given the suitable condition of desaturation, to build up a longer chain, additional double bonds being formed in the process. The classification of linoleic acid as a vitamin appears to us to be undesirable in view of the fact that linoleic and linolenic acids seem to be the necessary building stones for the synthesis of arachidonic and docosapentaenoic acids. The latter acid was also obtained from the livers of rats fed with the docosahexaenoic acid isolated from cod liver oil, so that in this the hydrogenation of one double bond must have occurred. This hexaenoic acid was shown [Hume *et al.* 1938] to produce no amelioration of skin symptoms; it is as yet unkown whether these are cured by arachidonic acid, since Turpeinen [1938], who carried out experiments on rats in which this acid was fed, gives only a general statement about the healing of skin symptoms.

It seems that whereas the essential linoleic or linolenic acids may have some direct effect on the skin, their main function is to supply material from which higher and more unsaturated acids may be synthesized. Sinclair [1935] has stressed the care with which these are guarded and the length of time that elapses before they are lost from the phospholipins which contain them, and has concluded that these are not, like other lipins, concerned in the transport of fat but play some special role in the animal metabolism. It is, however, of interest that in our negative controls where these acids were absent, the percentage of fatty acid in the livers was higher than in the livers where these more highly unsaturated acids were present. In the fat-deficiency disease there is an inability to store fat, but there is some evidence that fat may still be synthesized from carbohydrate; it seems possible that the highly unsaturated acids may play some part in the storage of fat, either by enabling the more saturated fats to be carried to the depots or by influencing its passage into the connective tissue cells in which it is stored. The study of the basal metabolism and respiratory quotients made by Wesson and Burr [Wesson, 1927; Wesson & Burr, 1931] lends some support to this view.

SUMMARY

The livers of rats fed on a fat-free diet were free from acids containing four or more double bonds; a hitherto unknown acid $C_{20}H_{34}O_2$ was isolated as its hexabromide, melting at 202–4°. Rats which had been kept for a long time on the fat-free diet and then fed with very small doses of methyl linoleate probably contained the same dihydroarachidonic acid as had been found in the negative controls. Arachidonic or some more highly unsaturated acid was also present and must, therefore, have been synthesized from the linoleic acid given.

Rats fed on a fat-free diet and then dosed with methyl linolenate (6 drops daily) synthesized an acid which, from the bromine content of its bromide, appears to have been a mixture of arachidonic and docosapentaenoic acids. The C_{20} trienoic acid was not detected. A fortnight after dosing with the C_{22} hexaenoic acid (prepared from cod liver oil) had ceased, the liver contained the C_{22} pentaenoic acid. Linoleic and linolenic acids appear to be the building stones essential for the production of more highly unsaturated acids which play some unknown part in enabling the animal to store fat in its depots and tissues.

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CCLXXXIII. THE ESTIMATION OF VITAMIN B_1 IN BLOOD

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MEIKLEJOHN [1937] has described a method, based upon the work of Schopfer [1935], of estimating vitamin B_1 in "small samples" (15 ml.) of blood. From his evidence he concludes "that the method...provides a quantitative estimate of the true vitamin B_1 content of the blood". Since a clinical test for vitamin B_1 in blood is badly needed, and since Meiklejohn's method is being used by several workers in this country and in others, it seemed to be important to examine the justification of his claim and the value of the test. The basis of the method lies in the ability of vitamin B_1 to promote the growth of a fungus, *Phycomyces blakesleeanus*.

There is already sufficient evidence contained in Meiklejohn's paper and in Schopfer's numerous publications to show that the former's claim needs investigation. Schopfer & Jung [1937, 2] have published tentative conclusions: "Le sang donne une réaction positive. En exprimant en aneurine la totalité de l'action auxogène observée, on arriverait à des teneurs variant entre 0·2 et 0·4γ par cm.^{3} de sérum." The animal used is not mentioned. In his other paper mentioning determinations on blood, Schopfer [1937] states: "Avec le sérum, nous avons trouvé une fois 39 mg. de récolte avec 1 ccm. (rat mâle) et une fois 69 mg. pour 1 ccm. (rat femelle); en comparant avec les données fournies par la vitamine pure, nous trouvons que 0·2γ de cette dernière livre une récolte de 65 mg." He then warns us of the danger of expressing all growth-promoting activity in terms of vitamin B_1, since "nous savons que d'autres facteurs agissent également". Although results on two rats hardly merit discussion, it is instructive to examine the only experimental results that he has published (presumably obtained with the male rat):

Rat serum vol. in ml.	Dry wt. of fungus mg.	Approx. value for apparent vitamin B_1 (γ/100 ml.) calculated from Schopfer's figures
0·5	34	17
1·0	39	10
2·0	47	6·5
Rat red blood corpuscles "about 2 ml."	77	15

It will be noticed that 0·5 ml. and 1 ml. serum give about the same actual growth; 0·5 ml. is nearly three times as effective as 2 ml. if the results are expressed per unit volume. These meagre results indicate that the method cannot be used for accurate quantitative estimations. They also indicate that rat serum contains an inhibitory factor.

Further, the method is not specific for vitamin B_1. Schopfer had great difficulty in identifying the growth-promoting factor owing to its great stability to heat, resistance to alkali and solubility in chloroform. Because of these

properties he at times discarded vitamin B_1 as the growth factor and supported bios [Schopfer, 1932], vitamin D, flavin [Schopfer, 1934, 1] and "factor M" [Schopfer, 1934, 2]. The only substances now known to promote the growth of the fungus are vitamin B_1 and its breakdown products; the pyrimidine and thiazole fragments (and certain closely related compounds [Robbins & Kavanagh, 1938]) are active when they are supplied together [Schopfer & Jung, 1937, 1; Sinclair, 1937; Robbins & Kavanagh, 1937. It seems probable that "factor M" consists of these fragments; and since heating (e.g. in cooking) may destroy the vitamin, these substances may appear in blood and so invalidate the test [Schopfer & Müller, 1938].

Meiklejohn found that the blood of avitaminous pigeons had an adjuvant action on added vitamin. This fact strongly condemns the validity of the test, and it will be shown below that normal blood has a similar action.

Although these facts cast doubt upon Meiklejohn's claim, it seemed advisable to examine the test experimentally.

Experimental

Unless otherwise stated, the technique of these experiments has only differed from that described by Meiklejohn in a few particulars: 0·4% asparagine[1] has been used unless otherwise stated; $MgSO_4$, $7H_2O$ in a concentration of 0·002 *M* was used instead of the anhydrous salt; the stock medium was brought to *p*H 6·5 with 10 *N* NaOH (the slight differences in *p*H produced by adding different amounts of blood did not affect the results), and was made up in stronger solution than used by Meiklejohn, so that only 4 ml. were added to each 50 ml. flask; 1 ml. spore suspension (containing about fifteen million spores) was used for inoculating. Since it has been found that the order in which the medium, blood, water and vitamin are added to the flask makes a difference to the results, unless otherwise stated the blood has been added to the medium and stood for at least half an hour; water (followed where necessary by vitamin) has then been added to the flasks and stood at $-2°$ for at least 12 hr. before sterilizing by steaming for 20 min. on 3 successive days. The cultures have been grown for 10 days in the dark in a room kept at a constant temperature of 18°. The stock culture of *Phycomyces blakesleeanus* (sex −) has been grown on Sabouraud's medium to which malt extract (B.P.) was added to make a concentration of 2%. Most experiments have been set up at least in duplicate and results quoted are from typical experiments. Synthetic vitamin B_1 has been used throughout.

Composition of medium

In the method described by Meiklejohn, different concentrations of asparagine were used for the control flasks and for those containing blood. He found that in presence of 2 or 3 ml. blood, there might be some reduction of growth if the concentration of asparagine were increased above 0·2%. This effect was said not to be observed with 1 ml. or less; one result with 1 ml. and none with less were quoted. The results with 2 and 3 ml. show an inhibition in the higher concentration of about 14%. Most of Schopfer's experiments were done with 0·1% asparagine, which is well below the optimum concentration. I have found that results with different concentrations of asparagine vary, but that 0·4% with blood usually gives a greater growth than is given by 0·2 or 0·6%. Even 0·4% is suboptimal in the control flasks without blood (Table I).

Asparagine was the best source of nitrogen that Schopfer [1934, 3] tried, although glycine was almost as good. I have tested various compounds, using amounts which supplied the same quantity of nitrogen as is present in 0·4% asparagine, and found that, as sources of nitrogen, guanidine sulphate, methyl

[1] B.D.H. asparagine and dextrose (A.R.) were used; the two inorganic salts were supplied by Kahlbaum ("puriss.").

Table I. *Effect of concentration of asparagine on the growth of* Phycomyces

In this and all the other tables, unless otherwise stated, the figures represent dry weight of fungus in mg.

		Concentration of asparagine (%)							
Exp.		0	0·1	0·2	0·3	0·4	0·6	0·8	1·0
1	0·1γ vit. B_1	0	—	30·8	—	41·0	38·4	40·2	—
	Ox blood 1 ml.	26·4	—	26·3	—	27·9	23·5	25·3	—
	,, 2 ml.	51·6	—	52·4	—	52·6	48·4	45·3	—
2	Ox blood 1 ml.	27·6	—	35·2	—	35·4	40·1	40·0	—
	,, 2 ml.	—	—	61·4	—	65·3	69·2	79·3	—
3	0·1γ vit. B_1	0	—	36·5	—	37·1	43·5	43·3	47·3
	0·5γ vit. B_1	0	—	73·8	—	101·4	115·6	120·7	132·3
4	0·5γ vit. B_1	—	—	—	—	101·0	113·8	—	—
	Ox blood 2 ml.	—	56·7	64·5	63·8	87·8	78·6	—	—
5	Ox blood 2 ml.	—	—	68·8	—	88·7	—	—	—
	+0·1γ vit. B_1	—	—	108·9	—	111·0	—	—	—
6	0·1γ vit. B_1	0	24·8	26·3	28·9	31·6	31·9	32·7	33·1
	0·5γ vit. B_1	0	38·1	60·0	66·8	93·7	100·9	101·2	98·4
	Ox blood 1 ml.	26·9	35·2	34·0	38·8	41·1	36·9	44·4	39·4
	+0·1γ vit. B_1	37·4	60·1	72·2	71·8	78·0	69·5	75·6	79·5
	+2·5γ vit. B_1	39·7	76·9	109·5	142·2	168·1	165·6	218·8	270·0
	Ox blood 3 ml.	43·9	66·4	86·3	92·4	100·2	93·4	97·5	97·2
	+0·1γ vit. B_1	52·6	81·4	104·2	110·7	126·9	115·8	124·0	128·2
	+2·5γ vit. B_1	59·5	88·5	135·6	169·7	195·5	219·3	233·5	309·3

guanidine HCl, dimethyl guanidine HCl, creatine and creatinine were useless; ammonium carbonate, urea and glycocyamine were very poor; alanine and arginine were poor; sodium aspartate and glycine were as good as asparagine, and "glutamine" much more effective (the glutamine was added direct to the medium, containing salts and glucose, before steaming; most of it will have been destroyed by this process).

Table II. *Comparison of the effect of different sources of nitrogen on the growth of* Phycomyces

		Source and % of nitrogen									
	Vit. B_1	Aspara-gine	Glycine			"Glutamine"					
Exp.	γ	0·4	0·2	0·5	0·75	0·2	0·4	0·6	0·8	1·0	1·2
1	0·1	46·0	46·0	50·7	53·8	43·8	53·2	56·2	—	—	—
	0·3	87·4	81·1	86·2	90·5	66·4	110·0	115·7	—	—	—
2	0·5	101·0	—	—	—	—	—	128·2	136·6	175·0	—
3	0·5	101·4	—	—	—	—	—	130·6	136·8	—	160·0

Glutamine was not quite as effective as asparagine when added to blood (Table III).

Table III. *Comparison of the effect of different concentrations of asparagine and "glutamine" in the presence of 2 ml. ox blood*

	Source and % of nitrogen				
	0·1	0·2	0·3	0·4	0·6
Asparagine	56·7	64·5	63·8	87·8	78·6
"Glutamine"	—	—	61·2	75·7	75·7

The most satisfactory source of nitrogen, either with or without blood, has been found to be hydrolysed casein.

100 g. caseinogen (Glaxo) were heated with 300 ml. conc. HCl and 300 ml. water under a reflux condenser for 3½ hr. After bringing to *p*H 6·5 with NaOH the total volume was 950 ml.

Table IV. *Comparison of asparagine and hydrolysed casein as sources of nitrogen for* Phycomyces

Exp.		Asparagine 0·4 %	Hydrolysed casein (%)							
			1	3	5	6	7	8	10	12
1	0·1 γ vit. B_1	37·1	—	—	—	58·7	58·7	60·3	58·0	45·3
2	0·1 γ vit. B_1	41·0	26·8	49·6	51·5	—	—	62·9	59·0	—
	1 ml. ox blood	27·9	—	53·1	55·8	—	54·1	66·3	63·3	—
	2 ml. ,,	52·6	—	86·3	105·0	—	102·6	110·8	82·1	—
	3 ml. ,,	70·0	—	81·7	86·7	—	88·0	84·3	81·8	—

Table IV and four other experiments have shown that with or without blood about 8 % is the optimum concentration of hydrolysed casein. The growth obtained with this concentration and 0·1 γ vitamin B_1 is about the same as is obtained with 0·4 % asparagine and 0·2 γ vitamin B_1. Without added vitamin, hydrolysed casein causes no growth of the fungus.

In Table IV the growth obtained with 3 ml. blood and hydrolysed casein is in general lower than that obtained with 2 ml. In Tables IV, V and VI a comparison is made of the growths obtained with and without blood or vitamin and the two different sources of nitrogen. From these and other similar experiments it is concluded that (i) in absence of blood, hydrolysed casein is a better source of nitrogen than asparagine; (ii) this result is also usually found in the presence of 1 ml. blood, although the estimated vitamin tends to be lower when hydrolysed casein is used; usually with 3 ml. blood, the estimated vitamin per unit volume of blood is less than with 1 ml. when hydrolysed casein is used, more than with 1 ml. when asparagine is used; (iii) when 0·1 γ vitamin is added to blood, a greater growth than expected is usually produced in presence of asparagine, and a smaller growth than expected is produced in presence of hydrolysed casein; (iv) this adjuvant action of blood in presence of asparagine is very marked when excess vitamin is added; but with excess vitamin and hydrolysed casein, blood sometimes has an adjuvant action and sometimes an inhibitory effect. This inhibitory action is being further investigated. These experiments prove that part of the adjuvant action of blood in presence of asparagine is due to sources of nitrogen in the blood. In this connexion it is interesting to recall that Meiklejohn found that extracting the vitamin from blood by means of alcohol or removing protein by heating in acid solution resulted in a considerable loss of growth-promoting activity.

It must be stressed that Schopfer has made no attempt to choose, and has not claimed to have chosen, the best medium for this fungus. The one employed by him and by Meiklejohn is likely to be suboptimal since it is poor not only in sources of nitrogen but also in salts ($MgSO_4$ and KH_2PO_4 are the only salts added by Schopfer). The addition of small amounts of various salts to the medium increases the growth; Czapek-Dox salts produce a better growth, and addition of $CaCl_2$, $FeSO_4$, $CuSO_4$, $MnSO_4$ and pyrophosphate, all increase it to varying extents, as shown in Table VII. In all cases pure salts were used; they were brought to *p*H 6·5 and added before steaming.

Table V. *Comparison of the effects of asparagine, hydrolysed casein and blood as sources of nitrogen for* Phycomyces

Source of nitrogen: O = no added nitrogen; A = 0·4% asparagine; HC = 8% hydrolysed casein

	Exp. 1 Human blood			Exp. 2 Ox blood			Exp. 3 Ox blood			Exp. 4 Human blood			Exp. 5 Human blood		Exp. 6 Ox blood	
Addition	O	A	HC	O	A	HC	O	A	HC	O	A	HC	A	HC	A	HC
0·1 γ vit. B_1	0	15·3	24·2	0	27·1	35·2	0	31·6	38·4	0	24·0	36·8	26·3	35·0	37·1	60·3
2·5 γ vit. B_1	0	114·5	157·0	0	110·8	180·2	0	93·7	186·4	0	66·8	172·8	113·0	126·4	101·9	—
1 ml. blood	18·6	19·1	28·2	28·0	37·7	41·2	26·9	41·1	29·4	18·8	22·6	26·3	25·1	33·7	27·1	42·3
+0·1 γ vit. B_1	32·0	44·6	43·0	36·1	66·1	66·5	37·4	78·0	49·4	—	63·1	—	48·0	—	—	—
+2·5 γ vit. B_1	72·9	129·0	206·0	40·9	149·0	81·0	39·7	168·1	91·2	—	135·0	—	—	—	—	—
2 ml. blood	—	—	—	38·6	73·4	57·7	—	—	—	—	32·2	—	48·6	—	45·5	55·2
3 ml. blood	52·1	69·5	63·8	41·1	89·0	78·0	43·9	100·2	64·3	—	50·3	—	63·0	65·0	—	—
+0·1 γ vit. B_1	63·5	90·8	81·8	—	—	—	52·6	126·9	77·6	—	—	—	—	—	—	—
+2·5 γ vit. B_1	128·5	201·0	224·0	—	—	—	59·5	195·5	102·4	—	—	—	—	—	—	—

Table VI. *Comparison of the effects of asparagine and hydrolysed casein as sources of nitrogen for* Phycomyces *in presence of blood*

Vit. B_1 γ	Ox blood (ml.) 0	1	2	3	4	5	6	7
	Hydrolysed casein (8%)							
0	0	29·4	63·6	64·3	113·5	118·2	124·5	132·1
0·1	38·4	49·4	75·4	77·6	116·3	123·4	137·1	—
2·5	186·4	91·2	99·6	102·4	136·5	163·7	169·9	—
	Asparagine (0·4%)							
0	0	41·1	66·0	100·2	118·6	122·5	—	—
0·1	31·6	78·0	93·1	126·9	128·7	—	—	—
2·5	93·7	168·1	165·7	195·5	171·9	—	—	—

Table VII. *The effect of salts on the growth of* Phycomyces

Exp. 1	0·3γ vitamin B_1	96·0
	0·3γ vitamin B_1 + $CaCl_2$ (52 mg.)	131·5
	0·3γ vitamin B_1 + sodium pyrophosphate (12 mg.)	116·2
Exp. 2	0·1γ vitamin B_1	46·0
	0·1γ vitamin B_1 + $CaCl_2$ (6 mg.)	53·4
	0·1γ vitamin B_1 + $CaCl_2$ (50 mg.)	65·4
	0·1γ vitamin B_1 + sodium pyrophosphate (1·7 mg.)	45·9
	0·1γ vitamin B_1 + sodium pyrophosphate (8·0 mg.)	53·4
	0·3γ vitamin B_1	87·4
	0·3γ vitamin B_1 + $CaCl_2$ (6 mg.)	104·1
	0·3γ vitamin B_1 + $CaCl_2$ (50 mg.)	126·2
Exp. 3	0·1γ vitamin B_1	37·1
	0·1γ vitamin B_1 + $FeSO_4$ (2 mg.)	41·1
	0·1γ vitamin B_1 + $CuSO_4$ (2 mg.)	50·0
Exp. 4	0·1γ vitamin B_1	39·5
	0·1γ vitamin B_1 + $MnSO_4$ (1 mg.)	40·0
	0·4γ vitamin B_1	83·0
	0·4γ vitamin B_1 + $MnSO_4$ (1 mg.)	95·6

These salts were supplied in larger amounts than would be present in the blood added. But since the medium selected is not a good one for the growth of the fungus, it would be expected that there might be substances present in blood that would have an adjuvant action upon it. Experiments now to be described show that such is the case.

Other substances in blood affecting growth

Meiklejohn concluded that the presence of blood does not usually alter the activity of the vitamin. The three lines of evidence that he advances are not convincing.

(1) He states that adding known amounts of vitamin to blood that has been autoclaved at *p*H 9 gives the expected growth, and the four figures he quotes support this. Yet this treatment, as he points out, is so drastic that it destroys other substances; for instance the reaction shifts during the process to *p*H 5. It is shown below that the adjuvant factor is thermolabile.

(2) He states that "the addition of a small amount of vitamin B_1 usually increases the growth of mycelium to the same extent in the presence of added blood as in its absence". Results on three samples of 2 ml. blood with and without 0·1γ vitamin are quoted in support of this. As already mentioned, he found that the blood from avitaminous pigeons had an adjuvant action on added vitamin and this I have confirmed. He stated that it was important to be aware of the possibility of blood containing an adjuvant factor, "and if necessary to control it" by including in the test a 2 ml. sample of blood to which 0·1γ vitamin had been added. He says that this gives a growth "considerably less" than the maximum, but his own figures show that it may be equal to the growth obtained with excess vitamin B_1.

As this point is important, I have examined it in some detail, and find that blood always contains an adjuvant factor. In experiments with ox blood summarized in Table VIII, known amounts of vitamin B_1 were added to the flasks and the total vitamin content was estimated. In some cases it was possible to estimate the apparent vitamin B_1 in the blood by subtracting the amount of vitamin B_1 added from the estimated total vitamin content; in such cases the apparent vitamin B_1 content, expressed in γ/100 ml. blood, has been printed in

Table VII in ordinary type. In other cases the amount of growth was greater than that obtained even with excess of added vitamin in the absence of blood, so that it was not possible to translate the weights of growth into apparent amounts of vitamin B_1; when this was the case the actual weights of growth have been given and have been printed in black type, as have also the maximum growths obtained with excess of vitamin alone.

The three experiments illustrated in Table VIII and many others show that adding small amounts of vitamin to ox blood produces in about 75 % of cases a

Table VIII. *Effect of added vitamin B_1 on the estimated vitamin in blood*

(For explanation of figures, see text.)

Vit. B_1	Ox blood (ml.)																
γ	0	0·5	1	2	3	4	5	0	1	2	3	4	0	1	2	3	4
0	0	11·0	8·0	9·0	10·5	**132**	**139**	0	10·0	11·0	**110**	**132**	0	7·5	8·0	13·5	11·5
0·025	—	—	—	—	—	—	—	—	11·0	13·0	**114**	**131**	—	—	—	—	—
0·05	—	9·0	10·0	10·5	9·5	**137**	**166**	—	11·5	19·0	**117**	**138**	—	9·0	11·0	—	—
0·1	—	6·0	8·5	9·0	9·5	**140**	—	—	16·0	**108**	**134**	**139**	—	11·5	16·0	—	—
0·15	—	8·5	13·0	8·5	—	—	—	—	31·0	—	—	—	—	—	—	—	—
0·2	—	15·0	18·0	7·0	**129**	—	—	—	**109**	**129**	**135**	—	—	—	—	—	—
Excess (2·5–5·0)	**118**	**136**	**132**	**147**	**157**	**175**	**205**	**105**	**132**	**143**	—	—	**104**	**166**	**204**	**266**	**278**
		Exp. 1							Exp. 2					Exp. 3			

greater growth than would be expected from the amount of vitamin added. The blood itself therefore usually has an adjuvant effect upon the growth of the fungus apart from the vitamin it contains.

Experiments with human blood have given similar results. In 150 1 ml. samples of blood from different cases the addition of 0·1γ vitamin B_1 has produced an increase over the expected of 50 % or more in 63 % of cases, an increase of less than 50 % or no difference in 30 %, and a diminution in 7 %. From this it appears that with small amounts of vitamin there is usually an adjuvant factor in blood, but occasionally an inhibitory factor.

One simple method of testing the validity of the method has not been used by Schopfer and has been disparaged by Meiklejohn. If there is no substance in blood other than vitamin B_1 which affects the growth of the fungus, then the growths obtained with and without blood when excess vitamin B_1 is added should be the same. They are not. In 50 experiments at least 2·5γ vitamin B_1 have been added to the flasks with and without the addition of different samples of human blood (1 ml.); this amount of vitamin is excessive because under the conditions of the experiments a maximum effect is obtained with about 0·5γ. The flasks containing the blood have always produced a growth that is greater than the control: the increase varies from 14 to 160 %, with an average for the fifty of 78 %. Further figures will be found in Tables I, V, VI, VIII and XI. Meiklejohn stated that "when large amounts (e.g. 0·4γ) of vitamin have been added [to blood] the effect has been less satisfactory. As noted previously, however, anomalous results are frequently obtained when the amount of vitamin is sufficient to produce nearly maximum growth." The size of the vessel and the accumulation of waste products of metabolism, amongst other factors, are suggested by him as probably explaining his inconsistent results. However, the results that I have quoted are consistent and significant and show that vitamin B_1 is not the only substance in blood that affects the growth of the fungus, since the addition of blood always produces a greater growth in presence of excess vitamin. An unusually large adjuvant action was shown by blood taken shortly after death

from a patient with aplastic anaemia; the following weights of fungus were produced:

	mg.
0·1γ vitamin B_1	25·8
2·5γ vitamin B_1	65·8
1 ml. blood	61·5
1 ml. blood + 0·1γ vitamin B_1	90·7
1 ml. blood + 2·5γ vitamin B_1	193·6

Meiklejohn's inconsistencies with nearly maximal growth are possibly partly due to insufficient drying of the mycelium before weighing; the mycelium takes a long time at 110° to reach a constant weight. Further, the adjuvant factor is thermolabile, and slight differences in the temperature at which the flasks are sterilized make considerable differences to the growth. With these precautions in mind, I have obtained consistent results.

Another fact that proves that blood has an adjuvant action is that large samples (more than 3 ml.) of blood (to which no vitamin has been added) usually give a growth that is greater than that obtained with excess vitamin B_1 (see Tables VI and VIII).

(3) The third line of evidence advanced by Meiklejohn to show that blood does not usually contain an adjuvant factor is that with separate samples of 1, 2 and 3 ml. blood the values obtained are in the ratio 1:2:3. He quoted six selected cases of human blood in support of this and states that he discarded the figures when this result was not obtained. I have found that there is usually an adjuvant factor present in blood so that the 3 ml. sample gives a value of more than three times the 1 ml. sample. Very rarely the blood has a depressant effect. Figures for ox blood have already been quoted. Some selected figures for human blood are as follows:

Table IX. *The effect of different volumes of blood upon the estimated vitamin in blood*

	Apparent vitamin B_1 in blood (γ/100 ml.)							
Ml. blood	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
1	13	8	6	7·5	8	6	10	13
2	18	18	11	15	12	12	9	6·5
3	**113** (5γ gives **106**)	**107** ($2·5\gamma$ gives **78**)	17	17	15	17	6	0

The figures in black type represent actual weights of fungus obtained (cf. Table VIII). Determinations upon samples of human blood from 235 different cases have given the following results: in 155 samples, the 3 ml. value (expressed in γ/100 ml. blood) has been 1γ, or more, higher than the 1 ml. value, and in 38 samples 1γ, or more, lower; in 213 samples the average 3 ml. value (expressed per unit volume) was 23 % higher than the average 1 ml. value, and in 22 samples the growth with 3 ml. blood added was greater than that given by excess vitamin. It must be admitted therefore that blood usually has an adjuvant action on the growth of the fungus; rarely there is an inhibitory action. This proves that Meiklejohn's method does not provide a quantitative estimate of the true vitamin B_1 content of blood.

The nature of this adjuvant factor is not fully known. It has already been shown that one cause of it is the additional sources of nitrogen, and possibly the salts, of blood. A further cause of it is to be found in the buffering power of blood. The fungus, in presence of excess vitamin, stops growing mainly because of the accumulation of products of metabolism. Although the medium is buffered

Table X. *The effect of calcium carbonate on the growth of* Phycomyces

A. Ox blood

Exp.		ml. blood 1	2	3	4
1	Blood alone	35·0	64·5	93·0	—
	+ $CaCO_3$	49·3	83·1	81·3	—
2	Blood alone	40·9	57·8	88·4	—
	+ $CaCO_3$	55·3	72·2	122·7	—
3	Blood alone	44·1	79·6	96·8	105·0
	+ $CaCO_3$	38·4	64·5	112·4	124·6
4	Blood alone	22·8	59·3	93·2	—
	+ $CaCO_3$	24·8	43·8	84·7	—
5	Blood alone	28·3	39·0	54·5	—
	+ $CaCO_3$	29·4	44·9	—	—
6	Blood alone	26·8	43·6	70·0	—
	+ $CaCO_3$	43·3	64·2	—	—
	+ $CaCO_3$ (added after steaming)	29·4	49·0	—	—
7	No vitamin B_1 { Blood alone	30·9	59·8	96·0	—
	{ + $CaCO_3$	36·3	57·5	88·9	—
	0·1 γ vitamin B_1 { Blood alone	59·3	82·8	110·4	—
	{ + $CaCO_3$	66·6	94·2	98·9	—
	2·5 γ vitamin B_1 { Blood alone	132·3	146·8	147·0	—
	{ + $CaCO_3$	122·7	136·3	39·8	—

B. Human blood (1 ml.)

Exp.		
1	Blood alone	20·5
	+ $CaCO_3$	31·0
2	Blood alone	19·2
	+ $CaCO_3$	22·8
3	Blood alone	29·8
	+ $CaCO_3$	40·0
4	Blood alone	26·1
	+ $CaCO_3$	40·2
5	Blood alone	27·6
	+ $CaCO_3$	21·8

C. Without blood

	Vitamin B_1 (γ) 0·0125	0·05	0·1	0·5	2·5	4·0
Control	—	—	22·9	—	—	103·9
+ $CaCO_3$	—	—	43·9	—	—	164·2
Control	—	17·7	32·1	73·5	107·1	—
+ $CaCO_3$	—	22·4	38·9	80·8	120·8	—

	Vitamin B_1 (γ) 0·1	0·2	0·3	0·5	2·5
Control	45·1	70	87·5	113	—
+ $CaCO_3$	48·6	78	103·5	123·5	—
Control	33·7	61·5	84·5	118	140
+ $CaCO_3$	51·4	75	133	162	285
Control	34·2	—	—	—	115
+ $CaCO_3$	47·0	—	—	—	161
+ $CaCO_3$ (added after steaming)	42·5	—	—	—	135·5

with phosphate, the solution has a *p*H of about 2 at this time. When blood is present the final *p*H is between 4 and 5. Blood therefore buffers the medium and allows the fungus to continue to grow for longer than it would otherwise do. This is undoubtedly one factor that contributes to the adjuvant effect. It can be tested by adding excess $CaCO_3$ (about 200 mg.). This does not alter the original *p*H of the medium; free acids produced by the fungus are neutralized by the $CaCO_3$ and the *p*H of the medium when the fungus has ceased to grow is still 6·5. When this is done it is found that a greatly increased weight of mycelium is produced (Table X c). The technique can be applied to blood, although the original *p*H must be carefully controlled, particularly if excess oxalate was used as an anti-coagulant. As a result of a number of experiments it has been found that the addition of $CaCO_3$ usually, but not always, produces an increase (averaging 16 %) in the growth produced by blood; but it invariably produces an increase (36 % on the average) without blood, showing that the buffering of blood is responsible for part of the adjuvant action (see Table X). This is very important, because Meiklejohn believed that over the steep part of the growth/vitamin curve (i.e. with concentrations of vitamin below about 0·3γ/10 ml.) "the limit to growth is determined solely by the amount of vitamin present"; the results summarized above show that his conclusion is not justified. It is interesting that pyruvic acid (isolated as the 2:4 dinitrophenylhydrazone) accumulates in large amounts in the flasks containing small amounts of vitamin (e.g. 0·1γ), but there is practically none in the flasks containing excess vitamin. This indicates that the vitamin may be concerned with the removal of pyruvate as much in the fungus as it is in the animal body.

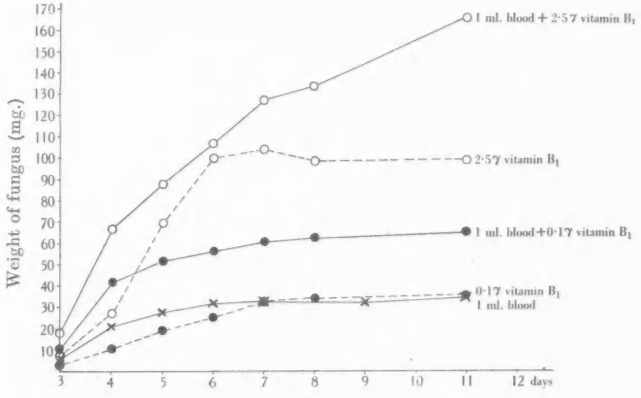

Fig. 1.

The growth produced in presence of blood differs in two further respects from the controls. First, the fungus in the flasks containing blood starts to grow more quickly than that in the control flasks containing approximately the same concentration of vitamin; the difference is particularly marked if vitamin be added to the blood (Fig. 1). Secondly, the ratio wt. of aerial hyphae/total wt. of fungus is much less in the flasks containing blood than in the control flasks containing approximately the same concentration of vitamin; and when excess vitamin is added to blood a very pale growth is produced which contrasts strongly

with the green hyphae in the control flasks with excess vitamin. These facts show that blood contains substances other than vitamin B_1 that affect the growth of the fungus.

There is one further piece of evidence that proves the existence of an adjuvant factor in blood. Meiklejohn stated that "some loss of growth-promoting activity has been found if the temperature has been raised above 110° or the time, at 107°, increased to more than 15 min." This presumably is meant to apply only in cases in which blood has been added, since the flasks without blood can stand autoclaving at higher temperatures for longer times without loss of activity. Vitamin B_1 is fairly stable at *p*H 6·5, and even if autoclaved for 10 min. at *p*H 8—which will undoubtedly cause some destruction—there is no loss in activity as Table III in Meiklejohn's paper shows. Schopfer & Jung [1937, 1] and Sinclair [1937] have indicated the reason for this, namely, that the vitamin is probably broken down to its constituent parts, and these can be utilized by the fungus. Schopfer & Müller [1938] have in consequence stated that it is necessary, when assaying vitamin B_1 with *Phycomyces*, first to adsorb the vitamin on fuller's earth and then to determine the vitamin in eluates made from this adsorbate. The loss of activity in the presence of blood which occurs on autoclaving at 107° (*p*H 6·5) for longer than 15 min. may be due to destruction of the vitamin but is more probably due to destruction of an adjuvant factor present in blood.

The results recorded in Table XI were obtained with human blood; the flasks were filled with medium and vitamin (where required), sterilized by steaming for 20 min. on three successive days and then filled under sterile conditions with the

Table XI. *The effect of heat upon the ability of blood to promote the growth of* Phycomyces

	Temperature (°)							
Addition	50	60	80	100	104·5	111·5	115·5	130·5
0·1 γ vit. B_1	—	—	—	15·3	15·0	15·5	15·2	12·3
2·5 γ vit. B_1	—	—	—	114·5	113·5	114·2	114·2	99·6
1 ml. blood	10·2	29·8	27·4	18·6	16·4	16·6	15·3	13·0
+0·1 γ vit. B_1	77·6	65·0	52·5	44·6	46·4	35·3	30·9	19·7
+2·5 γ vit. B_1	236·0	235·5	165·1	129·1	127·2	123·4	121·8	114·8
3 ml. blood	23·4	55·5	85·9	69·5	68·2	58·7	54·0	26·5
+0·1 γ vit. B_1	77·8	103·0	109·8	90·8	85·6	81·7	78·2	47·1
+2·5 γ vit. B_1	272·1	284·3	204·1	201·0	190·6	188·5	188·3	184·0

blood. The flasks were then heated at the required temperature for 10 min. I have already stated [Sinclair, 1938] that there is but little growth if the flasks containing blood are not heated because the vitamin in blood is bound; maximal growth is obtained after heating to about 60°; at higher temperatures there is a fall in growth due to destruction of adjuvant factor (shown by the fall in the flasks containing excess vitamin B_1). Since no change is produced by heating to 115° in the control flasks without blood, it is very important carefully to control the exact temperature in estimations; sterilizing at 110° for 10 min. will give a value about 15% lower than that obtained by sterilizing by steaming and 5% lower than after heating to 105°. This effect is also illustrated by Table XII.

Effect of storing blood

In any elaborate quantitative test, such as this, which is to be used for clinical purposes, it is often necessary to send samples of blood by post, or to keep samples until an experiment can be set up. (It may be mentioned that the

time consumed in the estimation on each sample of blood is about 2 hr. The test takes about a fortnight to complete.) Meiklejohn stated that oxalated blood will keep for "some hours" at room temperature without loss of vitamin; in presence of medium at $-2°$ it would keep "for days".

A few experiments have been done to test the effect of keeping the blood. The vitamin content was found to remain the same for about 6 days at $-2°$. If blood is kept for more than one week at this temperature the growth-promoting power usually increases owing to increase in adjuvant factor (see Table XII); this is probably due to autolysis producing compounds with adjuvant action. In seven experiments in which the blood was kept for between 8 and 24 days at $-2°$, the average increase in growth compared with the same sample tested at once was 32 % (16–50 %). In the experiment shown in Table XII human blood was drawn under sterile conditions. Some was added at once to the flasks containing medium etc. and sterilized next day (column I). Some was kept for 12 days at $-2°$ and then treated like the first sample (column II); it was also tested for sterility.

Table XII. *The effect of keeping blood upon its ability to promote the growth of* Phycomyces

		I	II
1 ml. blood	Unheated	—	8·5
,,	Steamed	31·0	40·6
,,	Autoclaved (110° for 10 min.)	27·4	36·5
1 ml. blood + 0·1 γ vitamin B_1	Steamed	60·0	58·7
1 ml. blood + 2·5 γ vitamin B_1	,,	106·0	160·0
2 ml. blood	,,	53·4	62·4
3 ml. blood	,,	63·4	78·3

Specificity of the test

It has already been stated that the method is now known not to be specific for vitamin B_1. In his paper Meiklejohn reported tests for four substances possessing growth-promoting activity for other organisms with negative results. Two of his observations I have been unable to confirm.

(1) Meiklejohn stated that a specimen of bios supplied by Mr J. R. O'Brien was without effect. I obtained specimens of bios from the same source and found that they had a remarkable effect (see Table XIII).

Table XIII

	mg.
Bios (0·1 ml.)	170
Bios (0·1 ml.) + 0·1 γ vitamin B_1	164
4·0 γ vitamin B_1	104

It will be seen that bios (1 ml. = 21 mg. solids = 500 "doses") had a very much greater effect than excess vitamin B_1.

(2) Mannitol was stated by Meiklejohn to have no effect on the growth of the fungus. This confirmed the previous report by Schopfer [1934, 3].

(3) Knight's staphylococcal factor was stated to have only negligible activity in amounts of 100 γ. Since Knight [1937] has shown that his factor consists partly of the breakdown products of vitamin B_1, and since three groups of workers [Schopfer & Jung, 1937, 1; Sinclair, 1937; Robbins & Kavanagh, 1937] have all found that these products are active in very small amounts, Meiklejohn's results are difficult to interpret. The remainder of Knight's factor is nicotinic acid or its amide; neither of these had any action on the growth of the

fungus. Coenzymes I and II, pure riboflavin and β-alanine were also inactive. A highly purified preparation of vitamin B_6 had no activity as a growth factor in the absence of vitamin B_1, but when added with the latter it doubled the growth.

In addition to bios, autoclaved "marmite", autolysed yeast and "Peters's eluate" all produced growths much greater than those obtained with excess vitamin B_1, even after they had been autoclaved at pH 9 to destroy this vitamin. I have already stated [Sinclair, 1937] that cocarboxylase is about as active as vitamin B_1, and under the conditions of the test the same is true of vitamin B_1 monophosphate. These results will be reported in detail later. It may be mentioned that several substances have been found that inhibit the growth of the fungus in small concentrations. For instance, 1 mg. indole in 10 ml. produces complete inhibition of growth.

Availability of vitamin in blood

In Meiklejohn's method, the corpuscles are allowed to settle into a thick mass at the bottom of the flask. Since he states that over 80% of the vitamin in blood is associated with the corpuscles it is important to ensure that it all becomes available for the fungus. Meiklejohn advances three lines of evidence in support of this. The first is not satisfactory because, as shown above, the value obtained with 3 ml. blood is usually more than three times that obtained with 1 ml. and therefore the smaller relative growth which would be expected in the larger sample if some of the vitamin were not available would be masked by the adjuvant action. The second is not fully convincing: in one experiment only, the corpuscular layer was apparently autoclaved twice, and this, Meiklejohn states, caused some loss of activity; in any case, the difference in the growths given by the surface layer (27·5 mg.) and whole blood (33·0 mg.) was not large. The third statement—that the corpuscular layer at the end of an experiment possesses no growth-promoting activity—is not surprising, since the fungus is known to produce an inhibitory substance during growth which would presumably be present in the corpuscular layer.

The availability of the vitamin in the corpuscular layer can be simply tested by comparing laked blood with blood in which the corpuscles have been allowed to settle. The results of 24 experiments show that there is no significant difference between the two methods and therefore Meiklejohn's method of allowing the corpuscles to settle out is fully justified.

Since in the control flasks the medium is liquid and in the flasks to which the larger samples of blood have been added it is partly solid, the effect of the fluidity

Table XIV. *The effect of the fluidity of the medium on the growth of* Phycomyces

A. Ox blood

	Vitamin B_1 (γ)		
	0·1	2·5	1 ml. blood
Control	30·7	117·5	20·7
With agar	43·2	142·5	31·3

B. Human blood

	1	2	3	4 ml. blood
Control	27·4	63·7	96·7	110·0
With agar	39·4	67·0	87·5	114·1

of the medium was tested by adding agar (50 mg.) to some flasks. The addition caused a slight increase in growth (Table XIV) although agar without added vitamin or blood produced no growth. It appears therefore that the fungus grows rather better on a solid medium, and part of the adjuvant action of the blood may be due to this fact.

Discussion

Williams, who originally [1919] suggested the use of yeast for the assay of vitamin B, has recently [1937] stated that "the use of fungi in quantitative testing for vitamin B_1 in extracts appears hazardous in the extreme". Orr-Ewing & Reader [1928] elaborated a quantitative test for vitamin B_1, using its growth-promoting activity with *Streptothrix corallinus*; the test was useful with fairly pure solutions of the vitamin, but interfering substances were present in cruder preparations. Heating in alkaline solution destroyed the vitamin, but some growth-promoting activity with the Streptothrix still remained [Peters *et al.* 1928]. Peters [1930] made the excellent suggestion that the micro-organism required the vitamin and could reactivate "alkalized" torulin. In these respects this micro-organism behaves like *Phycomyces*. In his earlier papers Schopfer realized that the fungus could only be used for the assay of fairly pure solutions of the vitamin. In his recent work he has become bolder and used the method with plant extracts, animal tissues, milk and blood; it has already been shown that his results on blood are not satisfactory. The fungus gives an appreciable growth with only $10^{-7}\%$ vitamin B_1, and there is no doubt that Schopfer's method is the most sensitive one for assaying pure solutions of the vitamin. For this purpose it has proved very successful [Faguet, 1937; Villela, 1938].

But blood contains other substances that influence the growth of the fungus; this is not surprising when it is remembered that it contains "phytotoxins" [Macht, 1936]. The method is not specific; according to Schopfer & Jung [1937, 2], *Phycomyces* "nécessité pour sa culture en milieu synthétique, la présence d'une constellation de facteurs de croissance de nature vitaminique parmi lesquels se trouve la vitamine B_1". Therefore Schopfer & Müller [1938] state that the vitamin must be adsorbed on fuller's earth before it can be assayed by this method. Moreover, it has been shown above that blood has an adjuvant action upon the growth of the fungus under the experimental conditions of the test. It was shown that addition of small amounts (about $0{\cdot}1\gamma$) of vitamin to blood usually produced a growth greater than would be expected from the amount of vitamin added, that adding excess vitamin to blood invariably produced a growth greater than the control without blood, that large samples of blood (greater than 3 ml.) usually gave a growth greater than that obtained with excess vitamin and that the adjuvant action was partly destroyed by heat and usually increased when sterile blood was kept at $-2°$ for more than a week. The adjuvant action is probably due to more than one factor; sources of nitrogen and salts in the blood, the buffering power of the blood and the more solid medium produced when blood is added, particularly in large amounts, probably all contribute to it. Under certain conditions, blood may be shown to have an inhibitory action on the growth of the fungus. Probably the inhibitory factor is normally masked by the adjuvant factor and is only apparent when the adjuvant action of blood is diminished by such devices as improving the sources of nitrogen in the medium.

These facts prove that blood contains substances other than vitamin B_1 that influence the growth of *Phycomyces* under the conditions of the test. Meiklejohn's conclusion cannot therefore be substantiated. I agree with Van Veen [1937] that in testing impure extracts (including blood and urine) by this method "the

results were far from reliable; in fact, that they were at times entirely unserviceable". Yet this is the only method available at the present time for attempting to assay vitamin B_1 in blood. If the sources of error mentioned in this paper are borne in mind and are controlled as far as possible, then the method is valuable for comparing the apparent vitamin B_1 in different samples of blood.

Summary

1. Following the work of Schopfer with the fungus *Phycomyces*, Meiklejohn described a method of estimating vitamin B_1 in blood. His conclusion that it provides a quantitative estimate of the true vitamin B_1 content of the blood has not been confirmed.

2. It is shown that under given conditions blood always contains substances that affect the growth of the fungus.

3. Hydrolysed casein is the most satisfactory source of nitrogen that has been found. By means of this it can be shown that sources of nitrogen in the blood affect the growth of the fungus even in presence of optimal concentrations of asparagine.

4. Under the conditions of the test the addition to blood of small amounts of vitamin B_1 usually produces a greater growth than expected, and addition of excess vitamin invariably does so.

5. The method is known not to be specific for vitamin B_1.

6. The importance is emphasized of carefully controlling factors such as the temperature of autoclaving and the length of time the blood is stored.

7. If the possible sources of error are borne in mind and controlled as far as possible, the method is valuable for comparing the apparent vitamin B_1 in different samples of blood.

I am deeply indebted to Prof. Peters for his interest and advice throughout this work. I am very grateful to Mr A. P. Meiklejohn for much help and advice. Mr H. W. Kinnersley and Mr J. R. O'Brien have kindly provided me with certain substances. To the Christopher Welch Trustees I am indebted for a grant.

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CCLXXXIV. WATER-SOLUBLE B VITAMINS

XI. THE ESTIMATION OF YEAST ELUATE FACTOR AND YEAST FILTRATE FACTOR BY RAT GROWTH METHODS

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In this laboratory it has been shown that, in addition to riboflavin, at least two other components of the vitamin B_2 complex are required for the nutrition of the rat [Edgar & Macrae, 1937].

One of these components we have named yeast eluate factor, since it was adsorbed from autoclaved yeast extracts by fuller's earth and was found in barium hydroxide eluates prepared from the fuller's earth adsorbates. This factor we recognized primarily as a growth factor for rats although we also found it curative of rat dermatitis. Dr Lepkovsky kindly put at our disposal a sample of his pure factor 1 [Lepkovsky, 1938], which is required by rats for growth and prevention of dermatitis [Lepkovsky *et al.* 1936] and is identical with crystalline vitamin B_6 [György, 1938; Keresztesy & Stevens, 1938; Kuhn & Wendt, 1938].

We have found that crystalline factor 1 completely replaces our yeast eluate fraction in the diet of the rat. We have also isolated from yeast a substance identical with crystalline factor 1 [Edgar, El Sadr & Macrae, unpublished] and thus the identity of our yeast eluate factor with factor 1 and vitamin B_6 is proved. The name adermin has been suggested [Kuhn & Wendt, 1938] for the crystalline material.

The other component we have named yeast filtrate factor since it was present in the filtrate obtained by fuller's earth treatment of autoclaved yeast extracts. This factor is required for the growth of rats and, as far as that property is concerned, corresponds with the factor 2 of Lepkovsky *et al.* [1936]. It is uncertain if our factor is identical with that required for growth and prevention of dermatitis in chicks fed on a diet of heated grains [Elvehjem & Koehn, 1935; Lepkovsky & Jukes, 1935; 1936]. Neither nicotinic acid nor nicotinamide could replace the yeast filtrate fraction in the diet of the rat [Macrae & Edgar, 1937].

The work carried out in this laboratory during the past 2 years, which has included the chemical investigation of these fractions and the preparation of large amounts for other biological investigations, has required that we should have reliable methods for the detection and estimation of these factors.

Rat growth tests have been successfully used in this laboratory for the determination of aneurin (vitamin B_1) and riboflavin (lactoflavin). As well as these members of the vitamin B complex, yeast eluate factor and yeast filtrate factor profoundly affect the growth of young rats [Edgar & Macrae, 1937], and we have found it comparatively simple to devise rat growth tests, similar to those used to determine aneurin and riboflavin, which have proved satisfactory for the determination of yeast eluate factor and yeast filtrate factor. These experiments are described in the present paper.

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Experimental

The general methods used in the tests for the B-vitamins have been described in previous papers from this laboratory [Chick *et al.* 1935; Edgar *et al.* 1937]. In the preparation of the litters of rats, the mothers received the full stock breeding diet except during the last week of lactation, when yeast was withheld. The number of rats in the litters was restricted to 8. The young rats, weaned at 21 days and weighing 40–50 g., received the basal diet consisting of casein 100, rice starch 300, cotton-seed oil 60, lard 15, salt mixture (McCollum's No. 185) 25 and water 500; the diet was cooked by steaming for 3 hr. Each rat also received from weaning daily supplements of 0·08 ml. cod liver oil and 10 μg. aneurin; when the body weight of the animals reached 100 g. the daily supplements of cod liver oil and aneurin were increased to 0·1 ml. and 15 μg. respectively.

In the preparation of vitamin B-free diets, the principal difficulty is to obtain vitamin-free protein of high nutritive value. We have found "Glaxo ashless extracted" casein to be satisfactory for certain investigations, although we have recognized that it is probably not free from B-vitamins. This casein was used in the earlier experiments reported in this paper, but later it was found advantageous to use a more highly purified casein.

Supplee *et al.* [1936] demonstrated that the washing of casein with a solution of NaCl removed riboflavin, and it is our experience that this process also removes other factors of the vitamin B complex from the casein. In the later experiments reported in this paper the casein used was purified by the following method.

"Glaxo ashless extracted" casein (2·5 kg.) was stirred for 30 min. with a solution containing 600 g. NaCl and 30 ml. glacial acetic acid in 30 l. tap water. The casein was allowed to settle for 1 hr. and the supernatant liquor was poured off. The washing with the salt solution was repeated 6 times. The casein was then pressed dry on Büchner funnels and stirred into 96 % alcohol (5 l.). The alcohol was removed by filtration and the casein spread in thin layers on the open bench and dried in a current of air.

The rats receiving the basal diet with supplements of cod liver oil and aneurin usually increased slightly in body weight during the first week after weaning, the average increase being about 4 g. for the period. The animals were then treated differently according to whether they were to be used in tests for eluate factor or filtrate factor. The animals were weighed 3 times weekly.

Preparation of supplementary materials

Yeast eluate fraction. The barium hydroxide eluate prepared from the fuller's earth adsorbate from autoclaved yeast extracts, purified by treatment with basic lead acetate, has proved thoroughly satisfactory. The method of preparation was described in a previous paper [Edgar & Macrae, 1937].

Yeast filtrate fraction. In the earlier experiments the filtrate fraction was obtained by simple treatment of an autoclaved yeast extract with fuller's earth by the method previously described [Edgar *et al.* 1937]. More recently we have purified this material by extraction with amyl alcohol, a procedure which has also proved effective in the purification of the factor required for the growth and prevention of dermatitis in chicks receiving a heated grain diet [Elvehjem & Kochn, 1935; Lepkovsky & Jukes, 1936]. The following method has been employed by us.

The fuller's earth filtrate (4 l., of concentration 1 ml. equivalent to 0·5 g. dry yeast) was concentrated in open trays at 37° to one-fourth of its original volume. H_2SO_4 was added to *p*H 1 and the extract was shaken with 2 l. amyl alcohol.

After separation, the amyl alcohol extract was shaken with 500 ml. water to which was added just enough NaOH to make the aqueous layer alkaline to thymol blue. The aqueous extract was separated, neutralized with HCl and the amyl alcohol again extracted with a second portion of alkali. The amyl alcohol was now transferred back to the yeast extract and the extraction repeated until the yeast filtrate had been extracted 6 times, the amyl alcohol being extracted with the alkali after each treatment of the yeast extract. The combined aqueous extracts of the amyl alcohol, containing the filtrate factor, were evaporated to small volume and treated with 4 volumes 96% alcohol. The precipitated salts were removed by filtration and the alcohol was distilled off *in vacuo*. A small amount of a gummy material which separated was discarded; in the final product 1 ml. was equivalent to 2 g. dry yeast.

Estimation of yeast eluate factor

Male rats were found more satisfactory for this test. They were prepared as described above and had received for 1 week from weaning the basal diet supplemented by cod liver oil and aneurin. For a period of 2 weeks the animals now received daily additional supplements of 50 μg. riboflavin and an amount of the yeast filtrate fraction equivalent to 2 g. dry yeast; these amounts were sufficient to supply the animals' requirements. An immediate growth response resulted and during the first week the animals usually increased in body weight by about 20 g. A slight fall in the rate of growth occurred in the second week during which the weight increase was usually about 15 g. Certain animals then received additional supplements of the materials to be tested for eluate factor activity, others (negative controls) were given no added supplement, while yet others (positive controls) were given an amount of a tested yeast eluate fraction equivalent to 2 g. dry yeast, this amount being known to supply the animals' requirements of eluate factor. The test was continued for a further 2-week period. Even after 2 days a sharp increase in the growth rate of the animals now receiving eluate fraction was noted, and the increased growth rate was maintained during the 2-week period, the average weekly gain usually being 25–30 g. (Fig. 1, Table I). The negative control animals generally continued to increase in weight at the rate observed towards the end of the preliminary period of 2 weeks, this rate being about half that of the positive control animals. Comparison of the growth rates of the animals receiving the test material with those of the negative and positive control animals indicated the vitamin potency of the tested material. As far as was practicable, animals used in a single test were taken from the same litter, although usually no great difference was observed from litter to litter. Reliable results were obtained when 3 animals, together with one positive and one negative control animal, were used for each test.

In the earlier experiment in which the basal diet contained unwashed "Glaxo ashless extracted" casein as source of protein and the unpurified yeast fuller's earth filtrate as source of filtrate factor, the slackening in the growth rate during the second week of the preliminary period was less marked than that observed when the more highly purified casein and purer filtrate factor preparations were introduced into the basal diet. The body-weight increase observed during the 2-week period following administration of eluate factor was also slightly less in the case of the animals receiving the purer diet; however, the growth response obtained when eluate factor was added to the diet was more striking and tests using the purified diet were generally more satisfactory.

It was observed that the increased growth rate resulting from the addition of eluate fraction varied with the dose given. In one experiment the average

total weight increase of 4 rats receiving a suboptimal dosage of eluate fraction was 40 g. for the 2-week test period, that of 5 rats receiving twice the above dosage was 49 g., while 4 rats given 4 times the dosage increased in body weight by 55 g. during the 2-week period. From this and other experiments not recorded it appeared that the growth response curve was the usual logarithmic one.

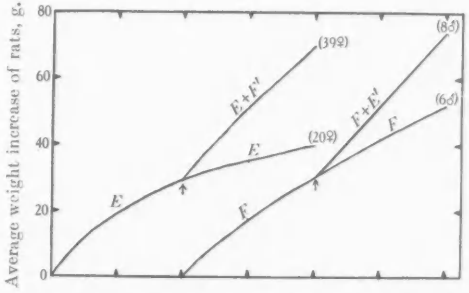

Fig. 1. Growth of young rats receiving a basal diet free from the B-vitamins, supplemented daily by 10–15 μg. aneurin, 50 μg. riboflavin and one or more of the following fractions: E = yeast eluate fraction (= 2 g. dry yeast), F = yeast filtrate fraction purified by amyl alcohol extraction (= 2 g. dry yeast), E' = eluate fraction from yeast (= 2 g. dry yeast), or liver (= 12 g. fresh liver), F' = filtrate fraction from yeast (= 2 g. dry yeast) or liver (= 6 g. fresh liver). The arrows indicate the points at which the doses were changed, and the figures and symbols in brackets the number and sex of the rats from which the growth curves were derived. Divisions on the abscissa indicate periods of 1 week.

Table I. *Tests for eluate factor*

Each rat (male) received daily 10–15 μg. aneurin and 50 μg. riboflavin

No. of rats	Filtrate fraction, preparation and amount given daily	Av. weekly wt. increase of group for preliminary period of 2 weeks g.	Additional supplement given during test period	Av. weekly wt. increase of group during test period of 2 weeks g.
Unwashed casein diet:				
52	Yeast filtrate fraction (= 1 g. dry yeast)	21, 19	Eluate fractions from yeast or liver	29, 25*
36	„ „	21, 18	None	16, 16†
4	Yeast filtrate fraction purified by extraction with amyl alcohol (= 2 g. dry yeast)	21, 14	Eluate fractions from yeast or liver	27, 27
Washed casein diet:				
9	Yeast filtrate fraction (= 1 g. dry yeast)	19, 16	„	27, 24
6	„ „	20, 19	None	15, 15
8	Yeast filtrate fraction purified by extraction with amyl alcohol (= 2 g. dry yeast)	16, 12	Eluate fractions from yeast or liver	22, 22
6	„ „	19, 13	None	12, 10

* The standard error of the average total weight increase for the 2-week test period ($\sigma/\sqrt{n}$) = 1·08.
† The standard error of the average total weight increase for the 2-week test period ($\sigma/\sqrt{n}$) = 1·29.

The unit of eluate activity we have adopted is based on the potency of an amount of our standard eluate fraction equivalent to 2 g. dry yeast. This amount, when given daily to a rat prepared as described above, produces a growth response of approximately 90 % of the maximum obtainable.

Estimation of yeast filtrate factor

A method very similar to the above has been used for the determination of yeast filtrate factor. Rats of both sexes, prepared as described above, having received the basal diet supplemented only by aneurin and cod liver oil for one week from weaning, received daily for 2 weeks 50 μg. riboflavin and a dose of yeast eluate fraction equivalent to 2 g. dry yeast. By the end of the first week the animals had generally increased in weight by approximately 20 g.; during the second week of this period, however, a very marked slackening in the growth rate occurred, and by the end of the week the growth rate was usually about 7 g. weekly, although over the whole of the second week weight increases of about 10 g. were generally obtained. Certain animals then received the added supplement of the material to be tested for filtrate factor activity. Negative control animals were given no added supplement, while positive control animals received either the yeast fuller's earth filtrate fraction from 1 g. dry yeast or the preparation of filtrate fraction purified by extraction with amyl alcohol, equivalent to 2 g. dry yeast. The test proper lasted for 2 weeks, and the presence of filtrate factor in the test material was indicated by an immediate increase in the growth rate, unmistakable even within 2–3 days. The growth rate increased to about 22 g. weekly, and this was maintained for the 2-week period (Fig. 1, Table II). The negative control animals usually continued to increase in body weight at the rate of about 7 g. weekly. Reliable results were obtained when

Table II. *Tests for filtrate factor*

Each rat received daily 10–15 μg. aneurin, 50 μg. riboflavin and yeast eluate fraction (=2 g. dry yeast)

No. of rats	Sex	Av. weekly wt. increase of group for preliminary period of 2 weeks g.	Additional supplement given during test period	Av. weekly wt. increase of group during test period of 2 weeks g.
Unwashed casein diet:				
6	♂	22, 12	Filtrate fractions from yeast or liver	25, 26
3	♂	19, 10	None	7, 5
39	♀	20, 10	Filtrate fractions from yeast or liver	22, 19*
20	♀	18, 10	None	6, 5†
Washed casein diet:				
10	♂	18, 11	Filtrate fractions from yeast or liver	21, 19
14	♂	19, 9	None	9, 6
7	♀	19, 8	Filtrate fractions from yeast or liver	19, 16
20	♀	18, 8	None	5, 4

* The standard error of the average total weight increase for the 2-week test period ($\sigma/\sqrt{n}$) = 0·88.
† The standard error of the average total weight increase for the 2-week test period ($\sigma/\sqrt{n}$) = 1·11.

3 animals, exclusive of controls, were used for each test. As far as possible animals were taken from the same litter but we found little variation between the litters. A certain difference existed between the males and females and this had to be considered in planning the tests. The growth response varied with the

amount of filtrate fraction given (see Table III). The tests in which the purer casein was used were generally more satisfactory, as the growth rate diminished more regularly during the second week of preparation when the animals were receiving the riboflavin and eluate factor supplements.

Table III. *Growth response of rats to graded doses of filtrate factor as contained in amyl alcohol extracts from* (*a*) *yeast filtrate fractions, and* (*b*) *liver filtrate fractions*

Each rat (female) received daily 10–15 μg. aneurin, 50 μg. riboflavin and yeast eluate fraction (=2 g. dry yeast)

	No. of rats	Av. weekly wt. increase of group for preliminary period of 2 weeks g.	Daily filtrate supplement given during test period	Av. weekly wt. increase of group during test period of 2 weeks g.
(*a*)	4	23, 12	Equiv. of 1 g. dry yeast	14, 20
	3	22, 11	Equiv. of 2 g. dry yeast	19, 22
	2	23, 13	Equiv. of 6 g. dry yeast	22, 18
(*b*)	2	17, 5	Equiv. of 3 g. fresh liver	11, 13
	2	22, 9	Equiv. of 6 g. fresh liver	23, 18

The unit of filtrate factor activity we have adopted is based on the potency of an amount of our standard filtrate fraction, purified by amyl alcohol extraction, equivalent to 2 g. dry yeast. This amount, when given daily to a rat prepared as above, produces a growth response of approximately 90% of the maximum.

Discussion

The rat growth method for the determination of factors of the vitamin B complex has been so extensively used in the past that the use of this criterion for the study of new factors of the vitamin B complex requires no further justification. In the case of aneurin, the rat growth method, although perhaps more tedious than some others, is certainly one of the more reliable biological methods for the determination of that vitamin. The biological determination of riboflavin has been almost exclusively carried out by rat growth methods; a paper published from this laboratory [Edgar *et al.* 1937] showed the regular growth response of young rats deprived of riboflavin to administered doses of that substance.

Although curative methods for vitamin determination have the advantage of specificity in cases where a specific group of symptoms is involved, growth methods which employ uniform healthy young animals appear to us to be the more reliable. Curative methods necessitate the use of sick animals and the response effected by administration of the missing vitamin is largely dependent on the degree to which the pre-existing deficiency had affected the general health of the animal.

For the estimation of vitamin B_6, which we now realize is identical with our yeast eluate factor and factor 1 [Lepkovsky *et al.* 1936], the cure of the dermatitis developed by rats deprived of the vitamin has been most extensively used [György, 1935]. In our laboratory, when using this method, we have encountered the disadvantages of curative methods referred to above. Further, we have also noted occasional spontaneous cures in our animals and have also found that the specificity of yeast eluate factor for the cure of this dermatitis is not complete [Chick & Macrae, unpublished]. Cures of dermatitis developed on diets deficient in yeast eluate factor and riboflavin, or deficient in yeast eluate factor and yeast

filtrate factor have frequently been obtained by the addition of riboflavin or of yeast filtrate factor, respectively. In carrying out the rat curative test, therefore, *the supply to the animal of all the other vitamin B factors must be adequate.* Since the animals used in curative tests are usually on experiment for many weeks, during which they must be carefully observed, the time required for these *tests is much greater than the simple routine rat growth test described.*

The only biological property of our yeast filtrate factor at present recognized is its effect on the growth rate of young rats. It is possible that the factor required by chicks for growth and prevention of dermatitis may be identical with this rat factor and, if this is found to be the case, the determination by the chick growth method [Lepkovsky & Jukes, 1936; Jukes & Lepkovsky, 1936] may prove a rival to the rat growth method.

We admit that the methods described in this paper for the determination of yeast eluate factor and yeast filtrate factor may be capable of improvement. For example, it might be found advantageous, especially in the case of the determination of yeast eluate factor, to extend the "running out period" by 1 week or more, in order further to reduce the growth rate immediately prior to adding the test material to the diet. The tests carried out as described above have, however, proved very satisfactory for the rapid testing of vitamin fractions obtained in experiments on the purification of the factors.

Summary

1. Rat growth methods are described for the determination of yeast eluate factor (vitamin B_6 of György; factor 1 of Lepkovsky) and yeast filtrate factor (factor 2 of Lepkovsky).

2. In both methods graded growth responses to graded doses of the factors were observed, and although the suitability of these tests for the accurate biological determination of these factors has not been worked out, the probability is that these methods, perhaps with some modification, will prove satisfactory.

We are very grateful to Dr H. Chick for her valuable advice and criticism, and we thank Messrs Hoffmann-La Roche for the gift of the riboflavin used in these experiments.

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CCLXXXV. THE WATER-SOLUBLE B-VITAMINS OTHER THAN ANEURIN (VITAMIN B_1), RIBOFLAVIN AND NICOTINIC ACID REQUIRED BY THE PIG

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From the results of previous work [Birch *et al.* 1937; Chick *et al.* 1938, 1, 2] it was concluded that young pigs could be reared successfully on a diet of maize and purified casein with the addition of a suitable salt mixture, provided that nicotinic acid were also given. Similar experiments made on rats showed that the addition of nicotinic acid was not required for this species [Chick *et al.* 1938, 2].

The fact that an extraneous source of nicotinic acid or its amide does not appear to be required by the rat is responsible for the misleading results of researches into the aetiology of pellagra in which this animal was used. The dog, the pig and the monkey appear to resemble more closely the human being in their requirement of nicotinic acid in their food and consequently in their failure to maintain health on diets consisting too largely of maize.

The vitamin requirements of different species of animals being so various, it seemed worth while to ascertain what other heat-stable, water-soluble, accessory factors in addition to nicotinic acid and riboflavin, are required by pigs. Whatever they may be, it is clear from our previous observations [Chick *et al.* 1938, 1, 2] that they are contained in whole maize in sufficient amount when it comprises 80% of the diet.

Edgar & Macrae [1937] have concluded that at least two other "B_2-vitamins" are necessary for rats. One of these they call "filtrate", the other "eluate" factor. Fractions containing these two factors were originally derived from yeast but Edgar *et al.* [1938, 2] have also separated similar fractions, using somewhat modified methods, from a water-acetone extract of liver.

In the present enquiry we are dealing with "filtrate fraction" and "eluate fraction" as described by Edgar and Macrae and prepared by their methods. Fractions derived by similar methods from yeast, liver and rice polishings have been prepared by Lepkovsky *et al.* [1936] and by other workers. The relation of these to those employed by us will be discussed later.

Experimental methods

It was first necessary to find a simple basal diet suitable for young pigs which would contain an adequate amount of vitamins A, D and E and of linoleic acid but which was as free as possible from water-soluble vitamins. To the basal diet a mixture containing vitamin B_1 (aneurin), riboflavin and nicotinic acid, all

given as pure chemicals, could be added as required. The following was found satisfactory:

Purified maize starch	71
Purified casein	21
Cod liver oil	2·5
Cotton seed oil	2·5
Salt mixture	3

It contained protein 18·1%, fat 6·0%, carbohydrate 64% and had a nutrient ratio of 1 to 4·2. As the pigs increased in weight the proportion of casein was gradually reduced to 15%. At first wood-charcoal was supplied as an unobjectionable form of roughage. This was found unnecessary and later discarded.

The maize starch was the purest sample obtainable and had a nitrogen content of 0·03%. It was prepared from dent white maize by the following process. The grain was steeped in a warm dilute solution of SO_2, then broken; the embryos were separated by flotation, the fibre by sieving and the protein by a process of differential sedimentation in an alkaline medium. Finally the starch deposit was washed by decantation until the nitrogen (protein) content was reduced to the required figure, after which it was filtered and dried rapidly in a current of dust-free air.

The cod liver oil was certified to contain per g. 1000 I.U. of vitamin A and 100 I.U. of vitamin D. The casein was "Glaxo ashless extracted" casein, described by the manufacturers as a casein of the "self soured" type, thoroughly washed with dilute acetic acid and afterwards extracted with hot alcohol; it contained 94% protein.

The salt mixture used was based on that recommended by Hubbell *et al.* [1937] who found it adequate for rats on a similar diet. It contained $CaCO_3$ 38%, bone ash 23·2%, NaCl 10·5%, KCl 27·2%, KI 0·008% and Fe_2O_3 0·84%. At first it formed 3% of the diet. Later it was realized that for rapidly growing pigs this mixture was low in phosphorus, the proportion of which in the diet, allowing for that in the casein, was less than 0·3%. The deficiency was rectified by mixing one-fifth of its weight of sodium dihydrogen phosphate (NaH_2PO_4, $2H_2O$) with that of the original salt mixture and adding 4% of the mixture to the diet. The phosphorus in the salt mixture was thus increased from 3·9 to 6·6% and that in the diet to 0·4%.

The riboflavin used was a sample of pure synthetic material kindly given by Messrs Hoffmann-La Roche, Basle, to whom we are also indebted for the specimen of nicotinic acid used.

The aneurin was pure synthetic hydrochloride provided by Prof. A. R. Todd.

The yeast was a commercial preparation of dried brewer's yeast.

Filtrate and eluate fractions prepared from liver

The laboratory preparation from yeast of the large amounts of filtrate and eluate fractions required in these experiments was not feasible and we had therefore to seek other sources. It was found by Edgar *et al.* [1938, 2] that fractions with similar biological properties, as tested on rats, were present in liver and were contained in the residues discarded during the commercial manufacture of some of the preparations used in the treatment of pernicious anaemia. These liver residues were kindly put at our disposal by Messrs Glaxo Laboratories, Ltd., who also prepared some of the fractions.

The liver residue available to us at the beginning of our experiment was the filtrate from the charcoal treatment of an aqueous acetone extract of liver.

Filtrate fraction was prepared from this by simple extraction of the acidulated liver residue with amyl alcohol, the filtrate fraction passing into the amyl alcohol. Eluate fraction was prepared from the material which had been extracted with amyl alcohol, by adsorption on fuller's earth and subsequent elution with barium hydroxide.

While the experiments were in progress the above liver residue became unobtainable, but two new residues were available. The first residue, that portion of an aqueous acetone extract of liver which was unextractable by phenol, was found to be a good source of eluate fraction and towards the end of our experiments we prepared an eluate fraction from this residue by adsorption on fuller's earth, elution with barium hydroxide and subsequent extraction of the eluate with amyl alcohol to remove any filtrate factor. The second residue, the filtrate from charcoal treatment of the phenol-extractable portion of the aqueous acetone extract, was rich in filtrate fraction and extraction of this residue with amyl alcohol gave the purified preparation of filtrate fraction which was used in the latter part of these experiments.

The amounts of eluate and filtrate fractions administered to the pigs were based on the requirements of rats found by Edgar & Macrae [1937] and Edgar *et al.* [1938, 1]. The animals in Exp. I (heavier pigs) received 100 "rat doses"[1] of filtrate fraction and 100 "rat doses" of eluate fraction daily. For the pigs in Exp. II we could not afford such a liberal allowance of eluate; they received 100 rat doses of filtrate and 50 rat doses of eluate daily. These allowances were chosen as being roughly proportional to the food intake of the pigs compared with that of the rats. At the beginning of the experiment each pig consumed about 50–100 times as much dry food as a young rat. The above allowances, however, were not increased or decreased as the pigs consumed more or less food during the course of the experiment.

The amounts given would appear to have been adequate, as 5 pigs (nos. 60, 62, 63, 65, 66) grew well when receiving both fractions. The dose of filtrate, at least, was probably not excessive, as pigs (nos. 60, 63) in which the dose of filtrate was reduced for 2 weeks showed an immediate diminution in growth, which was restored as quickly when the dosage was increased.

All the pigs received the basal diet and, except for those having yeast (see below), daily doses of aneurin 1 mg., riboflavin 2 mg. and nicotinic acid 25 mg. The doses of aneurin and riboflavin, like those of eluate and filtrate fractions, were calculated on the requirements of rats. The daily dose of 25 mg. nicotinic acid was adopted on the basis of previous experiments with pigs [Chick *et al.* 1938, 1] in which 60 mg. daily produced an immediate and striking effect in animals which had been depleted for 2–3 months; we decided that this dose would be excessive as a prophylactic. Sebrell *et al.* [1938] found 10 mg. nicotinic acid twice weekly an effective prophylactic dose to prevent black-tongue in dogs of about 6 kg. weight.

The pigs were divided into groups of 2–4 animals; one group received in addition to the above a daily dose of Edgar and Macrae's filtrate fraction, a

[1] The "rat doses" of filtrate factor and eluate factor, referred to in this paper, are equivalent to the units of these factors defined by Edgar *et al.* [1938, 1]. The unit of eluate factor is the potency of an amount of standard eluate fraction equivalent to 2 g. dry yeast and the unit of filtrate factor is the potency of an amount of standard filtrate fraction purified by amyl alcohol extraction, also equivalent to 2 g. dry yeast. When rats, whose growth rates are limited by deprivation of either filtrate factor or eluate factor, receive daily 1 unit of the appropriate missing factor, immediate growth responses occur of approximately 90% of the maximum.

second group received doses of their eluate fraction, a third group received both fractions and a fourth group yeast. The growth and health of these various groups were then studied.

General management of the animals

The general management of the animals has been previously described in detail [Birch *et al.* 1937]. The pigs receiving the same diet lived together in the same stall; these stalls consisted of commodious built-in rooms with concrete flooring and attached to each was a separate open run. The animals had no bedding, but a wooden platform was provided for them to sleep on. The diet was made up in batches of 50 lb. at a time; it was merely stirred with water to a thin cream and fed cold. The appropriate amounts of aneurin, lactoflavin, nicotinic acid and the various fractions for the whole group were stirred in immediately before feeding. The animals were fed twice daily up to the limit of their appetite. If any food was left at breakfast a corresponding diminution was made in their supper, so that all was consumed.

Experimental results

Experiment I

The initial object of this experiment was to ascertain what would happen to young pigs fed upon a diet of starch, casein, cod liver oil and cotton seed oil supplemented with (1) dried yeast and (2) aneurin, riboflavin and nicotinic acid. The experiment was begun on 2 May 1938. A litter of 8 pigs, 12 weeks old and of weights varying between 51 and 70 lb., was divided into 2 groups of 4 each.

The first group contained the lightest pigs (nos. 53, 54, 56 and 57). They had 4 % of dried yeast added to their diet and the casein and starch were correspondingly reduced. With the exception of no. 53, which died after 5 weeks and was found at autopsy to have a number of abscesses in the mesentery, these pigs made steady growth from the 2nd week onwards and gained an average of 10·9 lb. weekly. After 9 weeks the experiment was discontinued, as it was evident that 4 % yeast supplied all the B-vitamin requirements over this period (see Fig. 1).

The second group (nos. 50, 51, 52 and 55) received the same diet supplemented by aneurin, riboflavin and nicotinic acid. At first the animals grew well, but in all cases growth ceased abruptly after 3–4 weeks. These pigs did not manifest any symptoms of illness but it was clear that aneurin, riboflavin and nicotinic acid did not supply all that was required. From the 7th week the eluate fraction (100 rat day doses daily) was added to the diet of nos. 50 and 55 and the filtrate fraction (100 rat day doses daily) to that of nos. 51 and 52.

Pigs receiving eluate fraction

Unfortunately no. 55 developed an acute pneumonia shortly afterwards and died. At autopsy, extensive solidification of both lungs, purulent pleurisy and pericarditis were found. This pig cannot therefore be further considered.

Pig no. 50, after a resumption of growth which lasted 5 weeks, lost its appetite, suffered intermittently from diarrhoea with the passage of blood[1] and began to lose weight rapidly. The loss of weight continued although the diarrhoea abated. It was noticed to be weak in its hindquarters and moved with

[1] At autopsy this pig was found to have an infection of the mucous membrane of the large gut with the nematode *Oesophagostomum dentatum*.

a peculiar swaying gait. The weakness in the hindlegs increased until the animal had difficulty in maintaining equilibrium whilst eating or drinking and could not raise the hindlimbs sufficiently to scratch its body. At the end of the 14th week, that is 8 weeks after receiving the eluate fraction, the filtrate fraction was also added to its diet. A slight temporary improvement in appetite and general condition followed but the feebleness soon returned and the paresis of the hind quarters increased. Finally food was refused and the animal was killed.

Pigs receiving filtrate fraction

Pigs nos. 51 and 52 responded at once to the addition of filtrate fraction and for 1 month increased in weight at nearly the normal rate. At the end of this time growth ceased abruptly and during the next month No. 51 grew not at all and no. 52 but 7 lb. Both pigs then showed another spurt in growth which lasted for 3 weeks, during which each gained 20–25 lb. At the 10th week from the time they first received filtrate fraction growth again ceased in both of them (see Fig. 1). The first spurt of growth was understandable but the second was not. The latter coincided with the administration of a new preparation of filtrate fraction and ceased abruptly when that supply was exhausted. The most obvious interpretation would be that this particular preparation of filtrate fraction had been imperfectly fractionated, but we could not discover, by experiments upon rats, that it contained any eluate fraction. Pigs nos. 51 and 52 were occasionally observed to have epileptic fits.

Experiment II

This experiment, which was a continuation of the preceding one and overlapped it, was begun on 30 May 1938 with 11 9-week old pigs from a litter which had just been weaned; their weights ranged from 25 to 39 lb.

Two of the lightest pigs were used to find out how much dried yeast was needed to supplement the basal casein-starch diet. From the rate at which nos. 54, 56 and 57 of Exp. I were growing we concluded that 4 % in the diet was perhaps excessive. After 3 weeks on the basal diet, the diet of nos. 59 and 67 therefore had 2 % yeast added to it. The subsequent growth was, however, very poor, and after a further 3 weeks the yeast supplement was increased to 6 %. Immediate increase in growth rate followed and the average gain in weight soon exceeded 10 lb. per week. When this occurred the amount of yeast was reduced to 4 %, and the rate of growth was maintained. We conclude, therefore, that the amount of yeast required to supply all the B-vitamins for pigs of this age, on so simple a diet as that here used, is between 2 % and 4 % and is nearer 4 %.

The remaining 9 pigs of this litter were used to ascertain the effect of giving the filtrate fraction and the eluate fraction alone and in combination. As the pigs were a month younger than those of Exp. I it was thought likely that the effects might be more striking.

For the first 3 weeks all 9 pigs received the basal diet with the additions only of aneurin, riboflavin and nicotinic acid, in the same dosage as was employed with the first litter. This procedure was adopted as a measure of economy, for we had difficulty in making enough of the eluate and filtrate fractions to keep pace with the requirements of the animals and it was thought that depletion of their reserves during this preliminary period would abbreviate the experiments. The pigs showed a slow, subnormal rate of growth.

At the end of the preliminary period 3 pigs (nos. 58, 61 and 64) received the eluate fraction in daily amounts equivalent to 50 rat day doses, 3 pigs (nos. 60,

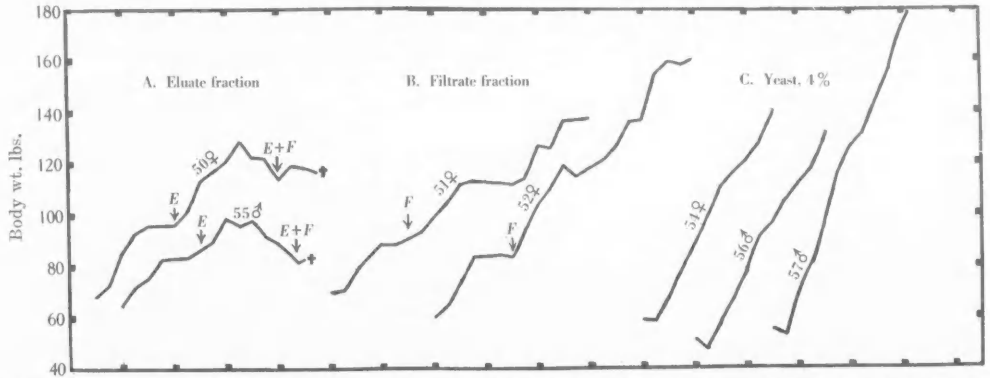

Fig. 1. Exp. I. Growth curves of young litter-mate pigs receiving a basal diet of purified casein, purified maize starch, cottonseed oil, cod liver oil and salt mixture, with daily rations of aneurin 1 mg., riboflavin 2 mg. and nicotinic acid 25 mg. Curves A, supplemented with 100 rat day doses of eluate fraction daily. Curves B, supplemented with 100 rat day doses of filtrate fraction daily. Curves C, basal diet supplemented with 4% dried yeast. *E* signifies addition of eluate fraction; *F* signifies addition of filtrate fraction; ✝ signifies death. Divisions on the abscissa indicate periods of 4 weeks.

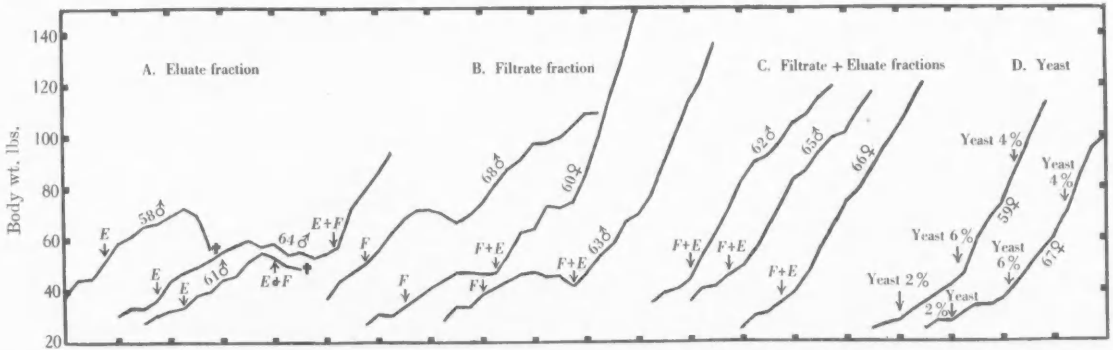

Fig. 2. Exp. II. Growth curves of young litter-mate pigs receiving a basal diet of purified casein, purified maize starch, cottonseed oil, cod liver oil and salt mixture, with daily rations of aneurin 1 mg., riboflavin 2 mg. and nicotinic acid 25 mg. Curves A, supplemented with 50 rat day doses of eluate fraction daily. Curves B, supplemented with 100 rat day doses of filtrate fraction daily. Curves C, supplemented with both fractions. Curves D, basal diet supplemented with dried yeast. *E* signifies addition of eluate fraction; *F* signifies addition of filtrate fraction; ✝ signifies death. Divisions on the abscissa indicate periods of 4 weeks.

63 and 68) received the filtrate fraction in similar dosage and 3 pigs (nos. 62, 65 and 66) received both fractions. The growth curves of all these pigs are shown in Fig. 2.

Pigs receiving eluate fraction only

The pigs receiving eluate fraction only (nos. 58, 61 and 64) grew subnormally for 6–7 weeks, the average weight increase being about 3 lb. weekly. Afterwards appetite failed and there was an abrupt cessation of growth and a decline in weight; nos. 58 and 61 died 2–3 weeks later. Shortly before growth ceased, it was noticed that all 3 pigs had weakness of the hind quarters and sagging of their backs. These symptoms increased day by day and the gait became unusual, the hind quarters swinging as they walked. It appeared as if the back end of the pig were loosely attached to the rest of the body. The hind legs were slightly flexed at all 3 joints so that the hind portion of the body was lowered. The clumsiness of the movements of the hindquarters was most noticeable when turning and increased day by day, the condition becoming progressively worse until, after about 10 weeks from the commencement of the experiment, nos. 58 and 61 were almost completely paralysed in their hindquarters. They were unable to raise them from the ground and if lifted could not remain standing. It was a flaccid palsy and at no time spastic.

For some weeks the animals pulled themselves about with their front legs dragging the collapsed hinder end of their bodies, which were rotated laterally. At this stage the deep reflexes were absent and the pigs appeared to be insensitive over the distal half of their hindlimbs, since pricking, either superficial or deep, elicited no response. The fore quarters and head and neck were unaffected. The pigs could still eat but had to be fed by hand. No. 61 was given filtrate fraction when it was severely ill. It became brighter and for a day or two its appetite improved, but it died a few days later.

The third pig in this group, no. 64, maintained its weight rather longer. The early symptoms of damage to the nervous system occurred at about the same time as in the others but did not develop to the same extent. This animal had the same swinging gait and clumsiness of the hindquarters and often fell when turning quickly, but it could always stand or get up on its hindlegs, perhaps after one or two unsuccessful efforts. The paresis of the left hindleg was greater than that of the right and the animal would stand on 3 legs, swinging it loosely back and forward. It remained in much the same condition for a month. 13 weeks after receiving the eluate fraction it was given the filtrate fraction also, in amounts equivalent to 200 rat doses daily. Improvement in the general condition of the animal occurred immediately. Its appetite returned and during the next week it consumed 3 times as much food as before and put on 15 lb. in weight (see Fig. 2). At the same time it became much stronger and learnt to make the best of its disablement. The paresis of the left leg and clumsiness in movement, however, remained.

Pigs receiving filtrate fraction only

The 3 pigs (nos. 60, 63 and 68) which were given filtrate fraction only, remained in better condition than those having the eluate fraction. The improved rate of growth which occurred when filtrate factor was first added, after the pigs had been 3 weeks on the basal diet alone, lasted 3–4 weeks. For the next month, however, weights remained stationary. Nos. 60 and 63 were then given the eluate fraction in daily amounts equal to 50 rat doses. Their appetite immediately increased and they started growing well, their weekly increase in weight steadily increased and in 6 weeks reached 15 lb.

No. 68 continued to have filtrate factor only and from 12 August to 5 September 1938 the same unaccountable spurt in growth occurred as was shown at the same time by nos. 51 and 52 of the previous litter (see above, p. 2211). We at first suspected the particular sample of filtrate fraction, but after that sample had been replaced by another, no. 68, after a stationary period, again resumed a moderate rate of growth (Fig. 2), suggesting that our suspicions were unfounded and that intermittent growth may be characteristic of deficiency of the eluate fraction.

None of the pigs deprived of the eluate factor seemed particularly ill but they all suffered from epileptic fits. These were first observed in no. 63, 7 weeks after the commencement of the experiment, when growth had just ceased. During the next week no. 60 was seen in a fit and 3 weeks later no. 68. It is impossible to say how frequently the fits occurred as the animals were not under observation during the greater part of the 24 hr.

Only one fit was observed in nos. 60 and 63 after they were receiving the eluate fraction as well as the filtrate, and that occurred on the day following administration of the former. In the case of no. 68, which was kept without eluate for 20 weeks, the epileptic attacks became more frequent, as many as 5 being observed in 1 week and 2 on 1 day.

The pigs receiving the filtrate fraction only were also noticed to have become pale. Estimations of haemoglobin in their bloods 2–4 weeks after they had ceased to grow (8 weeks after the beginning of the experiment) showed it to be less than 60% of the amount usually present in the blood of normally fed pigs of this age or in the blood of the pigs which were receiving yeast.

Pigs nos. 60 and 63 received the eluate fraction as well as the filtrate fraction from the 10th week onwards; the anaemia was steadily repaired and 8 weeks later their blood was nearly normal.

Details concerning the blood of all these pigs will be found on p. 2217, Table III below.

Pigs receiving both filtrate and eluate fractions

The 3 pigs (nos. 62, 65 and 66) which received both eluate and filtrate fraction at the same time as the preceding groups received one only of these fractions, remained healthy and grew steadily during the 11 weeks they were observed after these additions were made to their diets. They had no nervous symptoms and their blood remained normal. They did not, however, grow so well, although they ate as much, as those receiving yeast. Their average increase in weight per week was 7 lb. whereas that of the latter was 8·5 lb. It was also noted that their appetite diminished towards the end of the period whereas it should have become greater, having regard to their increased body weight.

We are able to compare the gain of weight per unit of food consumed by the pigs in these 2 groups over periods of 4 weeks, during which all the animals were of approximately the same weight. The pigs to which yeast was given gained 15·5 kg. and consumed 22 kg. of dry food. Those having both eluate and filtrate gained 16 kg. and consumed 29 kg. food. If 0·5 kg. of dry food per day be subtracted as the maintenance allowance for pigs of this weight—an amount representing the average amount of this diet required to maintain weight—the productivity, $\frac{\text{Wt. gained}}{\text{Food consumed} - \text{maintenance allowance}}$, was 1·4 for the diet with yeast and 1·0 for the diet with eluate and filtrate.

These facts suggest either that the supplements of aneurin, riboflavin, nicotinic acid, eluate fraction and filtrate fraction were not given in adequate amount,

or that, when combined, they do not possess quite all the nutritive virtues of whole yeast, a conclusion similar to that reached by Edgar *et al.* [1938, 3] from their experiments with rats.

Haematological observations

Anaemia from lack of mixtures of B-vitamins has been recorded in rats by Damianovich *et al.* [1923], Whitehead & Barlow [1929], Sure *et al.* [1931] and by György *et al.* [1937]; in pigeons by Barlow [1927] and by Hogan *et al.* [1937]; in dogs by Rhoads & Miller [1933], Spies & Dowling [1935] and by Fouts *et al.* [1938]; in monkeys by Day *et al.* [1935], Wills & Stewart [1935] and by Wills & Evans [1938]; in pigs by Miller & Rhoads [1935], Birch *et al.* [1937] and by Wintrobe *et al.* [1938].

The observations of Miller & Rhoads [1935] and of Wintrobe *et al.* [1938] are particularly pertinent to the present investigations because they concerned pigs, and those of Fouts *et al.* [1938] because they cured the anaemia with a fraction from rice polishings containing the "rat anti-dermatitis factor" (vitamin B_6). As will be seen below (p. 2221), Edgar and Macrae's eluate fraction which prevented or cured the microcytic anaemia we encountered in our pigs also contains vitamin B_6.

Systematic observations on the blood of the pigs were not carried out from the beginning of our experiments but as after 8–10 weeks on the deficient diets all the pigs were observed to be paler, determinations of haemoglobin and examinations of blood films were undertaken. These showed that a considerable degree of anaemia existed both in the animals deprived of eluate fraction and in those deprived of filtrate fraction and that the anaemia of the former was associated with the presence of large numbers of small corpuscles, 3–4 μ in diameter (microcytic). Henceforth, a more complete examination of their bloods was made from time to time. The observations are set out below in tabular form (see Tables I–III). Incomplete as they are, they permit of some definite conclusions concerning the effect on the blood picture of deprivation of Edgar and Macrae's filtrate and eluate fractions.

The blood was drawn from a vein either of the ear or of the tip of the tail. The haemoglobin determinations were made with a Sahli haemometer and checked with a Haldane CO haemoglobinometer. Fresh standards were prepared of such strength that the 100 mark on the tubes corresponded to 18·5 vol. O_2 or 13·8 g. haemoglobin per 100 ml. blood. The error of the pipette was ascertained by drawing human blood to the 20 c.mm. mark, allowing it to clot, weighing the pipette filled with blood and subtracting the weight of the pipette. The specific gravity of the blood was assumed to be 1·060. The graduated tubes were calibrated by adding approximately 1 and 2 g. water, weighing them and noting the levels in the tubes.

The corpuscular volume was ascertained by centrifuging bloods containing 0·2 % potassium oxalate in tubes of 1 mm. bore and 10 cm. length for half an hour at 4000 r.p.m. The proportion of the length of the column of corpuscles to that of the blood was multiplied by 1·1 to compensate for the shrinkage of the corpuscles due to this strength of oxalate [Wintrobe, 1931].

The mean corpuscular vol. in μ^3

$$= \frac{\text{Vol. corps.}}{\text{No. of corps.}} \text{ per } \mu\text{l.} \times 10^9.$$

Dr Lucy Wills very kindly measured the red corpuscles and plotted Price Jones curves from films taken from pigs nos. 61 and 64 which had been deprived

of filtrate fraction and from pigs nos. 63 and 68 which had been deprived of eluate fraction, and compared them with films taken from normal pigs of about the same age. The mean diameter, standard deviation and coefficient of variability arrived at from this laborious procedure, for which we cannot be too grateful, were:

No. pig	Mean diameter μ	Standard deviation	Coefficient of variability
Normal	5·87	0·48	8·2
61	5·83	0·69	11·8
64	6·25	0·49	7·9
63	5·64	0·69	11·8
68 (19. viii. 38)	5·16	0·98	18·9
68 (13. ix. 38)	5·54	0·84	15·9

The films supplied were poor ones, which perhaps accounts in some measure for the high standard deviation from the mean diameter. The changes in mean diameter are consistent with the results obtained by the haematocrit method, which by itself gives no indication of the variability in size of the corpuscles.

The data obtained from the examination of the blood of pigs nos. 59 and 67 will serve as a standard of reference (see Table I). These pigs taken from the same litter had been fed for 2 months on the basal diet supplemented by yeast; they were growing rapidly and were in excellent condition. The figures are close to some recently obtained by Mr Parry (personal communication) at the Institute of Animal Pathology, Cambridge, for healthy pigs somewhat younger than the present ones fed on an ordinary good diet. Mr Parry's data were obtained by methods precisely similar to ours. They also correspond closely with the averages of those found by Miller & Rhoads [1935] for their 15 young pigs before they received a deficient diet. We therefore assume that they may be taken as fair average values for normal pigs.

Table I. *Blood examination of normal pigs*

No. pig	R.B.C. millions per c.mm.	Hb, g. per 100 ml. blood	Corpuscular vol. %	Mean corpuscular vol. μ^3	Hb, g. per 100 ml. corpuscles	Remarks
I. Pigs nos. 59 and 67 after 10 weeks on basal diet supplemented by yeast; age 5 months; wts. 108 and 95 lb. respectively						
59	7·8	12·9	43	55	30	Films show little scatter in the size of cells; most appear to be about 6μ in diameter
67	8·1	12·4	44	54	28	
II. Pigs nos. 62, 65 and 66, after 3 weeks on basal diet and 10 weeks on basal diet supplemented by both eluate and filtrate fractions; age 5 months; wts. 115, 109 and 112 lb. respectively						
62	7·5	11·8	40	53	29	Most cells appear to be around 6μ in diameter
65	8·1	12·4	42	52	29	
66	8·0	12·9	45	56	28	

The effect of deprivation of filtrate fraction was to produce a moderate degree of anaemia in which both the number of blood corpuscles and the concentration of haemoglobin were reduced to about 2/3 of their normal amounts. The size of the corpuscles underwent little change. This type of anaemia is similar to that observed by Wintrobe *et al.* [1938] in pigs receiving minimal amounts of yeast. On receiving filtrate fraction, the composition of the blood of pig no. 64 (the only surviving pig in this group) showed little improvement after

Table II. *Effect on blood of deprivation of filtrate fraction*

Pigs nos. 58, 61 and 64. At the date (15. viii. 38) of the first observation they were 3 months old and had received the basal diet for 3 weeks, followed by 8 weeks on the same diet supplemented by eluate fraction; wts. 60, 50 and 60 lb., respectively. From 23. ix. 38 onwards no. 64 received filtrate fraction also.

No.	Date	R.B.C. millions per c.mm.	Hb, g. per 100 ml. blood	Corpuscular vol. %	Mean corpuscular vol. μ^3	Hb, g. per 100 ml. corpuscles	Remarks
58	15. viii. 38	—	7·9	—	—	—	Paralysed, losing wt.; died 17. viii. 38
61	15. viii. 38	—	7·0	—	—	—	Paralysed, losing wt.; died 25. viii. 38
64	15. viii. 38	—	9·6	—	—	—	Partial paralysis; wt. stationary
64	13. ix. 38	6·3	8·5	30	48	28	Films show abnormal scatter, mean diameter about 6μ
64	21. ix. 38	5·6	7·7	29	52	26	—
64	1. x. 38	5·4	6·9	26	48	26·5	Filtrate fraction also given from 23. ix. 38
64	11. x. 38	5·9	7·6	30	51	25	—

Table III. *Effect on blood of deprivation of eluate fraction*

Pigs nos. 60, 63, 68. At the date of the first observation (25. vii. 38) they were 4 months old and had received the basal diet for 3 weeks, followed by 5 weeks on the same diet supplemented by filtrate fraction only; wts. 47, 46 and 72 lb., respectively, and weight stationary. From 8. viii. 38 onwards nos. 60 and 63 were given eluate fraction also.

No.	Date	R.B.C. millions per c.mm.	Hb, g. per 100 ml. blood	Corpuscular vol. %	Mean corpuscular vol. μ^3	Hb, g. per 100 ml. corpuscles	Remarks
68	25. vii. 38	—	7·9	—	—	—	—
68	19. viii. 38	6·5	6·1	24	37	25	Numbers of small corpuscles of about 4μ diam.
68	13. ix. 38	7·0	5·8	24	34	24	—
68	21. ix. 38	7·1	4·2	23	32	17·5	—
68	11. x. 38	11·3	6·3	27	24	23·5	Numbers of small cells much increased
60	25. vii. 38	—	6·8	—	—	—	Amisocytosis: large numbers of small corpuscles of about 4μ diam.
63	25. vii. 38	—	7·1	—	—	—	
		From 8. viii. 38 nos. 60 and 63 received eluate also					
60	19. viii. 38	7·8	6·7	28	36	24	—
63	19. viii. 38	7·1	7·2	26	36	27	Still numbers of small corpuscles of about 4μ diam.
60	13. ix. 38	—	8·7	—	—	—	—
63	13. ix. 38	—	8·9	—	—	—	—
60	21. ix. 38	—	9·8	—	—	—	—
63	21. ix. 38	—	9·6	—	—	—	—
60	5. x. 38	8·8	11·7	37	42	31	Numbers of small corpuscles diminished
63	5. x. 38	8·3	12·0	33	40	36	—
60	15. x. 38	8·4	12·1	40	47	30	Few small corpuscles seen
63	15. x. 38	7·9	11·8	37	42	32	—

3 weeks, but this does not necessarily mean that its blood-forming tissue has not responded to the addition. Immediately after it received the filtrate fraction this pig started to grow at a great rate and added 25 lb. (45 %) to its body weight during the first 18 days and presumably the blood volume was also considerably increased (see Table II).

The effect of deprivation of eluate fraction was at first to cause a small decrease in the number of blood corpuscles and a considerable decrease in the size. In the case of pig no. 68, in which the deprivation was continued longest, the number of corpuscles increased later (11. x. 38) to about 50 % in excess of the usual number and their mean volume was reduced to about one half. The large proportion of small corpuscles ranging about 4μ in diameter was at once obvious on inspecting a dried film or when counting them in the haemocytometer. The amount of haemoglobin per ml. blood was half, and per unit of corpuscular volume three-quarters, of the normal, the result being a microcytic hypochromic anaemia. In those pigs, nos. 60 and 63, in which after 8 weeks the deprivation of eluate fraction was made good, the small corpuscles disappeared from the blood, slowly at first and then steadily, until after 7 weeks their size was nearly normal. At the same time the amount of haemoglobin increased and approached normality (see Table III).

Here again the initial slowness of recovery in the blood picture must again be considered in the light of the concomitant sudden increase in growth rate, and presumably in blood volume, brought about by restoration of the missing factor in the diet.

Comparison of our results with those of others leads us to conclude that there is more than one substance in the vitamin B_2 complex, the lack of which embarrasses the blood-forming tissues.

The anaemia observed by Wintrobe *et al.* [1938] in pigs fed on a synthetic diet not unlike that used by us, to which diminishing quantities of yeast were added as the experiment proceeded, was of the simple type which occurred in pigs nos. 58, 61 and 64 (see p. 2217, Table II above). These were the animals from which filtrate fraction was withheld. The number of red blood corpuscles and the amount of haemoglobin in their blood fell together without any obvious change in the dimensions of the red cells.

The anaemia in dogs suffering from black-tongue, produced by feeding them on Goldberger's pellagra-producing diet, was found by Rhoads & Miller [1933] to be of the macrocytic type. Later, when Miller & Rhoads [1935] turned their attention to pigs on a similar diet, they found the same evidence of increased activity of the bone marrow but the anaemia was microcytic in some animals and macrocytic in others.

Wills & Stewart [1935] produced a profound macrocytic anaemia by long-continued feeding of monkeys on polished rice, margarine, white bread and cod liver oil. Some success was obtained by treating the monkeys with Edgar and Macrae's filtrate fraction [Wills & Evans, 1938]. As it is the eluate fraction which prevents or cures the microcytic anaemia we have encountered, it is difficult to see the connexion between their experiments and ours. Nor is the precise relationship obvious between our observations and those of György *et al.* [1937] on aplastic anaemia in rats receiving a synthetic diet, beyond the fact that the anaemia was cured in both instances by an "impure preparation of vitamin B_6". Purified preparations of vitamin B_6, whilst curing the dermatitis characteristic of deficiency of this vitamin in rats, had no effect on the anaemia. In a later paper György [1938] concluded that the anti-anaemic substance was nicotinic acid. The anaemia in our pigs nos. 60, 63 and 68 deprived of eluate fraction,

was not aplastic in type and as it developed in spite of a daily ration of nicotinic acid, there does not appear to be much correspondence between these observations on rats and our own.

The recent observation of Fouts *et al.* [1938] that hypochromic microcytic anaemia, accompanied by convulsive fits, occurred in puppies maintained on a synthetic diet, do, however, afford an almost complete parallel with some of our experiments on pigs. Their basal diet included vitamins A and D, vitamin B_1, either as crystalline material or as purified concentrate, and riboflavin. The remaining constituents of the vitamin B_2 complex were provided as (1) a liver extract which contained the chick anti-dermatitis filtrate factor [Elvehjem & Koehn, 1935; Lepkovsky & Jukes, 1935; 1936] and nicotinic acid, and (2) an extract of rice polishings containing the "rat anti-dermatitis factor" (vitamin B_6). The activities of their preparations were controlled by experiments on rats and chickens. Four puppies which received the basal diet and liver extract only (Lepkovsky's filtrate factor + nicotinic acid) developed a severe anaemia and exhibited convulsive fits. The anaemia was cured when extract of rice polishings was added to the diet. Preparations (1) and (2) above correspond broadly to our "filtrate" and "eluate" fractions, respectively, except that in our preparations from yeast or liver the nicotinic acid or amide originally present in these sources was contained in the eluate fraction, being adsorbed together with it on fuller's earth at *p*H 1·3 and subsequently eluted, whereas in the preparation used by Fouts and his colleagues nicotinic acid was present in the filtrate after adsorption of their liver extract with Lloyd's reagent (see also below, p. 2222).

The results of the experiments of Hogan *et al.* [1937] are also difficult to bring into line, partly because no details are given. Pigeons were fed on a diet of casein, sucrose, cellulose and cod liver oil and, when polyneuritis had developed a concentrated preparation of vitamin B_1 was given. This cured the polyneuritis but about 2 months later the birds became profoundly anaemic. Neither riboflavin nor "anti-dermatitis concentrate" (?chick anti-dermatitis factor) was curative.

The anaemia occurring in pigs after gastrectomy, which has been studied by Bence [1933; 1934; 1936] and by Petri *et al.* [1937], should perhaps be mentioned, as this may be partly due to "conditioned deficiency", although the former author adduces evidence to show that it is caused by deprivation of Castle's intrinsic factor, which is manufactured in the mucous membrane of the normal stomach. According to Petri *et al.* [1937] the pigs manifested symptoms suggestive of pellagra and Bence's interpretation is not to be regarded as sufficient. An interesting pathological point is that, for the first year after the operation, the anaemia was of the hypochromic microcytic type associated with hyperplasia of the bone marrow. Bence reports that it subsequently became macrocytic and hyperchromic. The pigs studied by Petri and his colleagues were not under observation long enough for this result to be confirmed.

The confusion indicated in the précis of literature given above will not be cleared up until it is possible to employ pure substances in nutritive experiments in which one deficiency only is present. Even then it will probably be found that results obtained with one species of animal cannot necessarily be applied to another.

Nervous symptoms in pigs deprived of eluate factor and of filtrate factor

All the pigs in these groups had nervous manifestations; those deprived of eluate fraction had fits and those deprived of filtrate fraction became paralysed.

Altogether, some 30 fits were recorded and it is probable that more occurred when nobody was there to observe them. Their severity and frequency increased with the length of time the animals had been deprived of eluate fraction. They were typical epileptic fits as seen in the human being. The pig ran round in apparent terror, screamed (epileptic cry) and dropped as if shot, making no effort to guard the fall; tonic spasm followed during which the legs were extended. The pig became deathly white. This first stage was succeeded by a clonic stage, during which the limbs were jerked about violently; there was grinding of teeth and sometimes discharge of urine. The comatose stage followed; respiration was at first deep and stertorous, gradually becoming shallow. The pig looked as if dead but in a few minutes the colour improved, consciousness was recovered and the animal got up languidly and resumed his previous occupation, e.g. feeding. The duration of the fits was from a few minutes to a quarter of an hour. In the intervening periods the pigs appeared to be quite well. Minor epileptic manifestations were also observed in which the animals appeared dazed, butted into obstacles, tottered about without always actually falling and then rapidly regained consciousness. After the eluate fraction was added to pigs nos. 60 and 63 the epileptic seizures ceased.

In the experiments on puppies reported by Fouts *et al.* [1938] convulsions were associated with deprivation of a filtrate fraction somewhat similar to our own, prepared from liver; some of the puppies died during the fits. In experiments of earlier workers on dogs deprived of B-vitamins, convulsions were observed towards the termination of their lives, but as far as we know the only record of epilepsy being caused by a nutritive deficiency is one by Sheehy & Senior [1930], in a group of pigs deprived of vitamin D and shielded from ultraviolet rays by glass. The fits occurred from the seventh week of the experiment onwards and three of the animals died from them.

The paralytic symptoms which developed in the pigs deprived of filtrate fraction are described above (p. 2213) in the history of the animals in this group. It was a flaccid paraplegia confined to the hindquarters and accompanied by some disturbance of sensation. Once developed it was not cured by giving filtrate fraction (see pig no. 64).

Posterior paralysis of swine has been not infrequently encountered in young pigs in the United States. A good description of the disease was given by Wehrbein [1916] who found demyelination in the spinal cord and peripheral nerves. The symptoms described correspond closely with those observed in pigs nos. 58, 61, 64. Sporadic cases of posterior paralysis in pigs are also recorded by Doyle [1937]. Some of these occurred under conditions which rendered it improbable that the cause was nutritional in origin and Doyle therefore suggested that there was an infective form of the disease. The discovery of some scattered areas of small cell infiltration in the nerves and spinal cord supported this view.

Paraplegias have been reported in swine as the result of an inadequate supply of vitamin A by Hughes *et al.* [1928], Dunlop [1934], Eveleth & Biester [1937] and Foot *et al.* [1938]. The nervous symptoms observed by Dunlop were uncommonly like those seen by us, but they took a much longer time to develop. Deficiency of vitamin A can hardly have been responsible for the paralysis of our pigs which were deprived of filtrate fraction. Each pig received daily 25 g. or more of a reliable cod liver oil certified to contain more than 1000 I.U. vitamin A per g. The requirements of the pig for this vitamin were carefully determined by Dunlop [1935] who concluded that 6000 I.U. daily affords a 50 % margin for safety.

The recently published observations on pigs of Wintrobe *et al.* [1938] have a close bearing upon our own. The basal diet, consisting of purified casein, butter,

sucrose, cod liver oil and salts, was similar to that used by us. At first, 3 g. yeast per kg. body-weight were given daily, this dose being subsequently gradually reduced to 0·1 g. per kg. As the yeast was reduced, crystalline aneurin and riboflavin were given. After about 200 days the animals manifested nervous symptoms. These consisted of progressive ataxia, with some loss of deep and superficial sensation but without much motor weakness. Histologically there was severe degeneration of the posterior columns of the spinal cord, the dorsal ganglion cells and the peripheral nerves.

From a scrutiny of the diet it would appear that the pigs in these experiments of Wintrobe and his colleagues were gradually deprived of nicotinic acid and of the vitamins contained in our eluate and filtrate fractions. The symptoms were more of the sensory type than in our pigs, but further comment is deferred until the nervous systems of our animals have been examined by appropriate histological methods. Until that has been done we also postpone consideration of many interesting papers on the nervous lesions produced in other animals by feeding on diets deficient in some or all of what is conveniently called the vitamin B_2 complex.

Comparison of our eluate and filtrate fractions with those of other investigators

The fractions containing water-soluble B-vitamins other than aneurin and riboflavin which have been described by various investigators have mostly been prepared from aqueous extracts of liver, yeast and rice polishings by treatment with fuller's earth, but the conditions under which the adsorptions have been carried out have varied.

It is unlikely that the extracts of liver, yeast and rice polishings from which these fractions have been separated were nutritionally equivalent and it is quite possible, even should they be so, that the individual factors were present in the various extracts in different chemical combinations. The amount and composition of other materials present differ and affect the results of the adsorption processes. Further, fuller's earth is by no means a standard product, the adsorptive properties of different samples varying considerably. It is therefore not surprising to find that after fractionation of extracts of liver, yeast and rice polishings in various laboratories with the use of fuller's earth, the different essential nutrients are not always found in the corresponding fractions. For example, in the case of yeast, the fuller's earth adsorbate contains the greater part of the nicotinamide or other active pyridine base, as has been demonstrated in experiments on dogs and pigs, while in the case of some extracts of liver and of rice polishings these compounds have been found present in the fuller's earth filtrates. Similarly, filtrates obtained after treatment of liver extracts with fuller's earth are by no means nutritionally equivalent to those obtained by exactly similar methods from yeast when tested on the rat [Edgar *et al.* 1938, 2].

Comparison of the liver fractions used in this work with fractions prepared by others is therefore very difficult. When tested on rats, they had the same action as the filtrate and eluate fractions prepared from yeast by means of fuller's earth [Edgar *et al.* 1938, 2].

The eluate fraction certainly contained vitamin B_6, the rat anti-dermatitis factor of György [1935]. We found that the crystalline factor 1 of Lepkovsky [1938], which is identical with vitamin B_6, completely replaced our eluate fraction in the diet of the rat [unpublished results]. The rice polishings eluate used in the experiments on dogs by Fouts *et al.* [1938], in which a nutritional anaemia similar to that now observed in pigs was noted, would be similar to our liver eluate factor, in so far as they both contained vitamin B_6. It is

probable that our eluate fraction also contained nicotinamide [cf. Dann & Subbarow, 1938]. Since, however, our pigs received nicotinic acid in their basal diet, the possibility that nicotinamide was the essential nutrient supplied by the eluate fraction is excluded. Pigs deprived of eluate fraction were certainly deprived of vitamin B_6 but, until the experiments can be repeated with the use of crystalline vitamin B_6, the question whether all the symptoms observed are due to a deficiency of this vitamin must remain unanswered.

Our filtrate fraction apparently contained the nutrient required for growth of rats named factor 2 by Lepkovsky *et al.* [1936]. The growth-promoting action of their factor 2 for rats was very similar to that possessed by our liver filtrate fraction.

Lepkovsky & Jukes [1936] first gave the name "filtrate factor" to the substance obtained after treatment with fuller's earth of an extract of beef liver. This substance had a specific effect in the prevention and cure of the dermatitis described by Elvehjem & Koehn [1935] which occurs in chicks receiving a diet of yellow maize, wheat middlings and casein heated for 144 hr. at 100°. Lepkovsky & Jukes showed that their filtrate factor withstood autoclaving at *p*H 5·0, was not precipitated by lead acetate at *p*H 6·6 or by barium hydroxide, was not adsorbed on norite charcoal or lead sulphide and was extracted by amyl alcohol. Although in many respects our filtrate factor possessed similar properties to these, it is uncertain whether the chick "anti-dermatitis factor" which was also present in Lepkovsky's factor 2 and is possibly identical with the rat growth filtrate factor of Edgar and Macrae, would be contained in all the preparations of "filtrate fraction" used by us. From the properties described for this chick factor, the earlier batches used by us ought to have contained it, but the later batches made from phenol extracts would only have contained it if it is extractable by phenol. It is most improbable that our filtrate fraction would contain nicotinamide or other pyridine bases, since one step in its preparation was extraction from strong acid solution by amyl alcohol. The liver filtrate fractions used by Fouts *et al.* [1938] would probably contain all factors present in our filtrate fraction, and nicotinamide in addition. The relation of our fractions to the Elvehjem factor *W* [Elvehjem *et al.* 1936; Frost & Elvehjem, 1937] is obscure.

As was the case with our eluate fraction it is impossible to tell at present whether the vitamins in our filtrate fraction which are essential for the rat and the pig, respectively, are identical.

Summary

1. Pigs can be reared on a synthetic diet containing purified casein, purified starch, cottonseed oil, cod liver oil and a suitable salt mixture, and optimum growth can be obtained, when 4 %, but not 2 %, dried yeast is added.

2. When the above diet was supplemented by aneurin, riboflavin and nicotinic acid, growth ceased after 3–5 weeks according to the age and reserves of the animals at the beginning of the experiment.

3. If to the diet supplemented as described under 2 was added either the eluate fraction of Edgar and Macrae or their filtrate fraction, prepared from liver, growth proceeded at about one-third the normal rate, but was checked or arrested after 4–6 weeks.

4. When the eluate fraction was added to the diet of pigs which had previously received the filtrate fraction only and whose growth was arrested, there was an immediate and continuous gain in weight.

5. Addition of filtrate fraction to the diet of those pigs which had previously received only eluate fraction was followed by immediate improvement in appetite and rapid increase in weight in the one case in which paralysis was slight and the animal was not too ill to respond. The other 3 animals died in a few days after the filtrate was administered.

6. When, after about 3 weeks on the basal diet supplemented as described under 2, both eluate and filtrate fractions were given, there was an immediate response in food intake and good growth was observed for about 5 weeks, after which there was no further increase in appetite and the rate of gain in weight slackened accordingly. It is therefore probable that yeast contains some unidentified essential nutrient for the pig in addition to aneurin, nicotinic acid, riboflavin and what may be contained in the eluate and filtrate fractions.

7. Pigs maintained on the basal diet supplemented by eluate only, developed a flaccid palsy of the hindquarters.

8. Pigs receiving supplements of filtrate only, developed a microcytic anaemia and had typical epileptic fits. On receiving supplements of the eluate the blood returned to normal and the fits ceased.

9. In addition to aneurin, nicotinic acid and probably riboflavin, at least two further water-soluble vitamins are necessary for the nutrition of the pig. These vitamins are contained in the filtrate and eluate fractions of Edgar and Macrae; whether the whole activity of the eluate fraction is to be accounted for by the vitamin B_6 which it contains, cannot be decided until this vitamin is available in the pure state in sufficient quantity to be tested on pigs.

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Note added 7 December 1938. Since the above paper was sent to press we have received the report of work by Hughes [1938, *Hilgardia*, **11**, 595], on the requirements of the pig for different members of the vitamin B complex. Although his basal diet and supplements were not as highly purified as those used by us, his observations were made on more animals, 83 young pigs in all; the results are in general agreement with our own. Vitamin B_1, riboflavin given as whey adsorbate, nicotinic acid and "rice bran filtrate" (Lepkovsky's factor 2) were all needed to supplement a basal diet of polished rice, purified casein, salt mixture and cod liver oil. The whey adsorbate, given as source of riboflabin, doubtless contained our "eluate factor". The growth observed with the above supplements was superior to that occurring when 3 % yeast was added to the basal diet. This is in agreement with our observations indicating 4 % yeast as the minimum required to ensure an adequate supply of all the B-vitamins.

CCLXXXVI. THE WATER-SOLUBLE B-VITAMINS

XII. THE PREPARATION FROM LIVER OF FRACTIONS CONTAINING THE SAME DIETARY ESSENTIAL FACTORS FOR THE RAT AS YEAST ELUATE FRACTION AND YEAST FILTRATE FRACTION

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In this and certain other laboratories yeast has been employed as a source of the B-vitamins, whilst some workers have favoured the use of liver. Similar experimental methods have been used for the fractionation of liver and yeast extracts, and of these adsorption with fuller's earth has been most generally employed. We have obtained by this means two fractions from autoclaved yeast extracts containing distinct rat dietary essentials. These we have called yeast eluate factor and yeast filtrate factor [Edgar & Macrae, 1937].

The identity of yeast eluate factor, vitamin B_6 [György, 1935] and factor 1 [Lepkovsky *et al.* 1936] is established [Edgar, El Sadr & Macrae, unpublished]; the relationship of the yeast filtrate factor to similar factors described by other workers is discussed in the preceding paper [Edgar *et al.* 1938].

Fractionation of liver by the methods we used to separate the factors present in yeast extracts did not yield fractions with biological properties corresponding to those of the yeast fractions. However, by other methods we have obtained fractions from liver extracts biologically similar to the yeast eluate and filtrate fractions. The present paper describes these experiments.

Experimental

In general the rat growth method used in the testing for yeast eluate and yeast filtrate factors has been employed [Edgar *et al.* 1938]. The preliminary preparation of the rats was carried out as described, the young animals receiving for the first week after weaning the same basal diet and daily supplements of 0·08 ml. of cod liver oil and 10μg. of aneurin. Thereafter, the experimental methods were varied to some extent, as is described in the separate experiments.

Yeast eluate fraction and yeast filtrate fraction were prepared by the methods described in the previous paper [Edgar *et al.* 1938]. All the liver fractions were prepared from liver residues obtained in the manufacture of the pernicious anaemia factor [Laland & Klem, 1936]; these were kindly supplied to us by Messrs Glaxo Laboratories. Three residues have been investigated.

Liver residue I. This residue was the filtrate resulting when a liver extract, obtained by extraction of liver with aqueous acetone and subsequent removal of the acetone, was treated with charcoal. Experiments with rats showed that this residue probably contained all of the factors of the vitamin B_2 complex with the exception of riboflavin, since rats receiving the basal diet supplemented by cod

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liver oil, aneurin, riboflavin and this liver residue grew as well as did rats receiving that diet with whole yeast or liver extract as the source of the B_2-vitamins.

Liver residue II. The liver extract made by aqueous acetone extraction of liver, freed from acetone and reduced to small volume was extracted with phenol. The fraction insoluble in phenol was liver residue II. This liver fraction was also rich in members of the vitamin B_2 complex, although it contained less of the liver factor corresponding to yeast filtrate factor than did liver residue I.

Liver residue III. The aqueous layer, obtained when the above phenol extract was shaken with water and ether, was treated with charcoal. Liver residue III was the filtrate from the charcoal. This residue contained considerable amounts of the rat dietary factor corresponding to the yeast filtrate factor and only traces of other factors.

Liver residue I has been mainly used in this investigation.

Fractionation of liver residue I with fuller's earth

The liver fraction (1 l.; 1 ml. = 5 g. fresh liver) was adjusted to *p*H 1·2 by the addition of H_2SO_4, and 50 g. fuller's earth ("specially selected, activated", Fuller's Earth Union) was added. After stirring at intervals for 30 min. the adsorbate was removed by filtration. The adsorption was repeated, and the filtrate, after treatment with $Ba(OH)_2$ to remove H_2SO_4, was ready for administration to rats.

The first adsorbate was washed twice with 500 ml. of *N*/10 HCl and suspended in 700 ml. of 2 % $Ba(OH)_2$. After several hours at 0°, the eluate was removed by filtration, and the adsorbate was again eluted with 500 ml. of 2 % $Ba(OH)_2$. The combined eluates, freed from Ba with H_2SO_4, were adjusted to *p*H 8 and treated with an excess of basic lead acetate (8 g. in 40 ml. of H_2O). The resulting precipitate was filtered off and the excess lead removed from the filtrate with H_2S. The PbS was filtered off and the filtrate reduced *in vacuo* to 250 ml. (1 ml. = 20 g. fresh liver).

Young rats, prepared as described, having received the basal diet and the cod liver oil and aneurin supplements for 1 week, were each given additional daily supplements of 50 μg. of riboflavin and either 1 ml. of the liver fuller's earth filtrate (= 5 g. fresh liver) or 1 ml. of liver fuller's earth eluate (= 20 g. fresh liver).

The animals receiving the liver fuller's earth filtrate gained in weight at an average rate of 30 g. weekly for 3 weeks (Table I). This growth rate is nearly double that obtained when rats receive the diet supplemented by yeast fuller's

Table I. *Effect of fractionation of liver residue I with fuller's earth*

Each rat (male) received daily 10–15 μg. aneurin and 50 μg. riboflavin

No. of rats	Daily supplement given during period of 3 weeks	Av. weekly wt. increase of group during 3-week period (g.)	Additional daily supplement given during 4th week	Av. weekly wt. increase of group during 4th week (g.)
2	Fuller's earth filtrate from liver residue I (=5 g. fresh liver)	35, 29, 27	Fuller's earth eluate from liver residue I (=20 g. fresh liver)	37
2	Fuller's earth filtrate from liver residue I (=5 g. fresh liver)	33, 28, 29	None	26
4	Fuller's earth eluate from liver residue I (=20 g. fresh liver)	22, 15, 10	Yeast fuller's earth filtrate not purified by amyl alcohol extraction (=1 g. dry yeast)	25

earth filtrate (see Table II), indicating that the liver filtrate contains other dietary essentials for the rat in addition to those present in yeast filtrate fraction. The addition of liver fuller's earth eluate fraction to the diet after 3 weeks did, however, cause an increase in the growth rate, showing that the liver fuller's earth filtrate was deficient in a growth factor for rats which was contained in the liver fuller's earth eluate fraction.

The rats which received the liver fuller's earth eluate fraction from the end of the first week after weaning gained less in body weight than those receiving filtrate. Here again, however, the growth rate was somewhat in excess of that occurring after administration of yeast eluate (see Table II), suggesting the presence of other dietary essentials in this liver fraction. The addition of yeast filtrate fraction to the diet caused a striking increase in the growth rate, which proved that the dietary essential contained in the yeast filtrate fraction was a limiting factor in this liver fuller's earth eluate fraction.

The above procedure therefore, which is essentially the same as that which was employed in the separation from yeast of the yeast eluate and yeast filtrate factors, did not yield fractions from liver with the same biological properties as those from yeast.

Preparation from liver residue I of fractions biologically similar to the yeast filtrate and yeast eluate fractions

Since fuller's earth fractionation of liver extract did not yield fractions corresponding to the yeast fractions, other methods of separation had to be employed. The liver preparations described below with activities similar to those of the yeast filtrate and eluate fractions we have named liver filtrate fraction and liver eluate fraction, respectively.

Liver filtrate fraction. Liver residue I (500 ml.; 1 ml. = 10 g. fresh liver), adjusted to *p*H 1 with H_2SO_4, was extracted six times with 800 ml. portions of amyl alcohol. The combined extracts were then shaken three times with 1 l. portions of water containing enough NaOH to make the aqueous layer alkaline to thymol blue. The combined aqueous extracts were neutralized with HCl, evaporated *in vacuo* to about 100 ml. and treated with 4 vol. of 96% alcohol. The precipitated salts were filtered, and the filtrate, after removal of alcohol *in vacuo*, was adjusted to 800 ml. (1 ml. = 6 g. of fresh liver).

Liver eluate fraction. To the residue from the amyl alcohol extraction of liver residue I diluted with 3 vol. of water (now 1 ml. = 2·5 g. fresh liver) and readjusted to *p*H 1·2, 150 g. of fuller's earth were added. After stirring for 30 min., the adsorbate was collected on a Büchner funnel and washed thoroughly with *N*/10 HCl; it was then twice eluted with 1 l. and 500 ml. portions of 2% $Ba(OH)_2$. To the combined eluate, freed from Ba with H_2SO_4 and adjusted to *p*H 8 with NaOH, an excess of basic lead acetate (25 g.) was added. The resulting precipitate was filtered, and the lead removed from the filtrate with H_2S. The filtrate was then reduced in volume *in vacuo* to 200 ml. (1 ml. = 25 g. fresh liver).

Comparison by the rat growth method, of the eluate and filtrate fractions from liver with those from yeast

The rats used in these growth tests were prepared in the usual manner. After the usual depletion period of 1 week during which they received supplements of cod liver oil and aneurin, each male rat was given the additional daily supplements of 50 μg. of riboflavin and either yeast filtrate fraction purified by amyl alcohol extraction equivalent to 2 g. dry yeast or the above liver filtrate fraction equivalent to 6 g. fresh liver; each female rat received riboflavin and either yeast

eluate fraction equivalent to 2 g. dry yeast or the above liver eluate fraction equivalent to 12 g. fresh liver.

The two groups of male rats receiving the filtrate fractions increased in weight at approximately the same rate during the 2-week period during which they received this diet (see Table II). The average weight increases of the animals in

Table II. *Comparison of eluate and filtrate fractions from liver with those from yeast*

Each rat received daily 10–15 μg. aneurin and 50 μg. riboflavin. The supplements indicated in the table were given daily in the following equivalents: liver filtrate fraction = 6 g. fresh liver; liver eluate fraction = 12 g. fresh liver; yeast filtrate fraction, purified by amyl alcohol extraction = 2 g. dry yeast; yeast eluate fraction = 2 g. dry yeast.

No. of rats	Sex	Daily supplement given during preliminary period	Av. weekly wt. increase of group for preliminary period of 2 weeks g.	Additional daily supplement given during subsequent period of 2 weeks	Av. weekly wt. increase during subsequent period of 2 weeks g.
2	♂	Liver filtrate fraction	19, 13	Liver eluate fraction	32, 23
2	♂	Liver filtrate fraction	22, 20	Yeast eluate fraction	27, 26
5	♂	Yeast filtrate fraction	17, 14	Liver eluate fraction	25, 24
6	♂	Yeast filtrate fraction	18, 12	Yeast eluate fraction	24, 25
8	♀	Liver eluate fraction	16, 10	Liver filtrate fraction	25, 27
2	♀	Liver eluate fraction	22, 12	Yeast filtrate fraction	22, 21
10	♀	Yeast eluate fraction	20, 10	Liver filtrate fraction	24, 18
6	♀	Yeast eluate fraction	21, 13	Yeast filtrate fraction	22, 21

the two groups receiving the eluates from yeast and liver for the 2-week period were also the same. Certain of the rats of the two groups which had received the different filtrate fractions were now each given an additional daily supplement of yeast eluate fraction (= 2 g. dry yeast) and the others were given the liver eluate fraction (= 12 g. fresh liver). In all cases a marked increase in the growth rate resulted, and all animals continued to increase in weight at approximately the same rate during the 2-week test period, irrespective of the sources of filtrate and eluate fractions in their diets.

The rats which had received the eluate supplements during the first 2 weeks were given supplements of the filtrate fractions; certain animals of the two groups were each given doses of yeast filtrate fraction (= 2 g. dry yeast) and the others were given the liver filtrate fraction (= 6 g. fresh liver). Again, rises in the growth rates of all animals occurred, the weight increases being the same whether the animals received their fractions from yeast or liver.

The above experiments prove beyond reasonable doubt that liver filtrate and liver eluate fractions, prepared as described, contain the same dietary essentials for the rat as the fractions prepared from yeast.

Preparation of liver eluate and filtrate fractions from other liver residues

Liver eluate fraction from liver residue II. The liver residue II was treated with fuller's earth without previous extraction with amyl alcohol; the adsorbate was eluted with $Ba(OH)_2$ and the eluate purified by treatment with basic lead acetate as described above. When tested on rats this fraction proved to be contaminated with filtrate factor, which, however, was easily removed by extraction of the preparation with amyl alcohol. The product had then the same growth-promoting properties for rats as the yeast eluate fraction.

Liver filtrate fraction from liver residue III. Amyl alcohol extraction of liver residue III by the method described for the preparation of liver filtrate fraction from liver filtrate I, yielded a preparation with the same growth-promoting properties as the yeast filtrate fraction.

Discussion

Considerable confusion in the literature concerning the vitamin B complex has resulted from the indiscriminate use of yeast, liver, rice bran, wheat germ and other materials as sources of factors in the complex. Fractions prepared in the same way from different materials have sometimes been finally proved to have quite different biological properties. Confusion has also been caused by certain authors assuming that effects noted in different experimental animals after deprivation of the same fraction are due to lack of the same factor. As each additional factor in the vitamin B complex is discovered, the need for caution in generalizing conclusions drawn from such results becomes more apparent.

In this laboratory we have realized for some time that the dietary essentials of the vitamin B complex contained in yeast fuller's earth filtrates are by no means always the same as those contained in liver fuller's earth filtrates. Experiments on pigs [Chick *et al.* 1938] and dogs [Koehn & Elvehjem, 1936] have proved that, while yeast fuller's earth filtrates contain little or no nicotinic acid or other pyridine derivative with similar biological properties, liver fuller's earth filtrates are rich in these substances. The marked difference, reported in this paper, between the fuller's earth filtrates as sources of rat dietary factors, cannot be due to the difference in the amounts of nicotinic acid derivatives they contain, since we have not been able to show that nicotinic acid or its derivatives have growth-promoting properties for rats [Macrae & Edgar, 1937]. Lepkovsky *et al.* [1936] reported that the preparation of liver fuller's earth filtrates free from factor I (yeast eluate factor) was comparatively easy, but it is possible that in our experiments all of that factor was not removed from the liver filtrate. Further biological investigation of liver fuller's earth filtrate fraction must be carried out before the relationship of the dietary factors contained in it to those of other vitamin fractions is clarified; the proof of the biological identity of one fraction from liver with our yeast filtrate fraction is one step in that direction. Since extractability from acid aqueous solution is a common property of the rat filtrate factor and the chick antidermatitis factor [Elvehjem & Koehn, 1935], the probability that these factors will eventually prove identical is increased. The relationship between our rat dietary factors and those required for growth and maintenance of pigeons [Carter & O'Brien, 1937] is an interesting problem which will probably be solved in the near future.

Only when all factors of the vitamin B complex are obtained in a pure state will the vitamin B problem be finally solved. The isolation of yeast eluate factor (vitamin B_6, factor I) in a pure state [Lepkovsky, 1938; Keresztesy & Stevens, 1938; György, 1938; Kuhn & Wendt, 1938; Edgar, El Sadr & Macrae, unpublished] has proved an important step. Since the crystalline vitamin completely replaces our eluate fraction in the diet of the rat, it seems probable that vitamin B_6 is the only essential nutrient for the rat present in our yeast and liver eluates. The possibility, however, still exists that other essential nutrients may be common contaminants of our eluate fraction, our filtrate fraction and even our basal diet.

SUMMARY

1. Fuller's earth treatment of liver extracts did not yield fractions with the same dietary essentials for the rat as did similar treatment of autoclaved yeast extracts.

2. Fractions with the same growth-promoting properties for rats as the yeast eluate fraction and yeast filtrate fraction were obtained from liver extracts by other methods.

We are very grateful to Dr H. Chick for her valuable advice and criticism. We wish to thank Messrs Glaxo Laboratories, Ltd. for gifts of large amounts of liver extracts and Messrs Hoffmann-La Roche for gifts of riboflavin.

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CCLXXXVII. THE ISOLATION OF A FLAVOPROTEIN FROM COWS' MILK

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The starting-point of this investigation was the observation that concentrated solutions of the xanthine oxidase of milk were characterized by a pronounced orange coloration. We have been able to isolate a flavoprotein compound which accounts for some though not all of this colour. There are, very likely, other flavoprotein compounds in milk besides the one to be described.

I. *Isolation*

(1) Fresh untreated cows' milk is warmed to 35° and treated with rennet as described by Dixon & Kodama [1926]. The curd is broken up and filtered through muslin. The filtrate is mixed with 1·1 vol. of sat. $(NH_4)_2SO_4$. The precipitate rises to form a sharp layer, and the fluid underneath is sucked off and discarded. The layer of precipitate is filtered with suction on Büchner funnels. The well-packed cake of precipitate is dried *in vacuo* over H_2SO_4 and the fat is extracted with ether. The yield is *ca.* 700 g. from 100 l. of milk. The powder keeps indefinitely when stored dry.

(2) A 10% solution of the powder is cooled to 0° and mixed with 0·56 vol. of sat. $(NH_4)_2SO_4$. Glacial acetic acid is added with vigorous stirring until the *p*H is 4·0 (just yellow to bromocresol green). The precipitate is centrifuged, and mixed with water equal in volume to the discarded supernatant fluid. After neutralizing with NaOH, the total volume is measured and the degree of saturation of the solution with respect to $(NH_4)_2SO_4$ is calculated on the assumption that the cake of precipitate is 36% saturated. 0·1 vol. of ethyl alcohol is added with vigorous stirring followed by sufficient sat. $(NH_4)_2SO_4$ to bring the degree of saturation to 25%. After acidification with glacial acetic to *p*H 4·0 the precipitate is centrifuged and discarded. The supernatant fluid is made 50% saturated with $(NH_4)_2SO_4$ and the precipitate filtered with suction and dried *in vacuo*. The yield is *ca.* 200 g. from 100 l. of milk.

(3) A 5% solution of the above powder is dialysed against running tap water for 12 hr. and mixed with 0·05 vol. of 25% basic lead acetate solution. The bulky precipitate is rapidly centrifuged and discarded. The relatively clear supernatant fluid is mixed with an equal volume of sat. $(NH_4)_2SO_4$. The centrifuged precipitate, which contains $PbSO_4$ and protein, is resuspended in 5 vol. of water. After neutralizing with NaOH, the suspension is centrifuged. The supernatant fluid which is retained has a pronounced orange colour. After dialysis against running tap water until free of $(NH_4)_2SO_4$, the solution is mixed with an equal volume of ethyleneglycol monoethyl ether and the *p*H adjusted to 3·8 by addition of glacial acetic acid. The precipitate is centrifuged and redissolved in the minimum quantity of *N*/100 NaOH. The solution is dialysed against running tap water and finally

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against distilled water until free of the reagent. The yield at this stage is about 2 g. from 100 l. of milk.

(4) At 38 % saturation of $(NH_4)_2SO_4$ and *p*H 7·0 only part of the flavoprotein is precipitated together with a large amount of colourless material. The precipitate is discarded. The supernatant fluid is made 50 % saturated with respect to $(NH_4)_2SO_4$ and centrifuged. The above procedure is repeated twice. The orange-red precipitate is dissolved in the minimum volume of *N*/100 NaOH and the solution dialysed against distilled water until salt-free. The yield is *ca.* 0·25 g. from 100 l. of milk.

(5) Milk flavoprotein is readily adsorbed on alumina Cγ at *p*H 6–7 and can be eluted from the adsorbing agent with a mixture of $(NH_4)_2HPO_4$ and NH_3 according to the method of Weygand & Stocker [1937]. This method of purification is only successful after extensive concentration of milk flavoprotein. The minimum quantity of alumina necessary to adsorb flavoprotein completely from a 0·5 % solution is used and this quantity must be determined experimentally for each preparation. Two elutions are sufficient for extraction of at least 90 % of the adsorbed flavoprotein. The combined eluates are dialysed against distilled water. After two adsorptions the yield is *ca.* 0·12 g. from 100 l. of milk.

Table I contains a summary of the scheme of purification and of the degree of concentration at the various stages. Assuming a 95 % loss in the purification

Table I. *Preparation of milk flavoprotein*

	Flavoprotein present in
Milk	
↓	
Rennin treatment, 35°	Filtrate
↓	
50 % sat. $(NH_4)_2SO_4$	Precipitate
↓	
Dried and extracted with ether	Precipitate
↓	
38 % sat. $(NH_4)_2SO_4$ *p*H 4·0 and 0°	Precipitate
↓	
25 % sat. $(NH_4)_2SO_4$ *p*H 4·0 and in 10 % alcohol	Filtrate
↓	
50 % sat. $(NH_4)_2SO_4$	Precipitate
↓	
Precipitate dried and dialysed (0·01 % flavinphosphate)	—
↓	
5 % solution treated with 0·05 vol. 25 % basic lead acetate	Filtrate
↓	
50 % sat. $(NH_4)_2SO_4$	Precipitate
↓	
Precipitate redissolved and dialysed after centrifuging off $PbSO_4$	Filtrate
↓	
Treated with equal volume ethyleneglycol monoethyl ether (0·1 % flavinphosphate)	Precipitate
↓	
Redissolved precipitate dialysed and 38 % sat. $(NH_4)_2SO_4$ at *p*H 7·0	Filtrate
↓	
50 % sat. $(NH_4)_2SO_4$ (0·2 % flavinphosphate)	Precipitate
↓	
Repeat twice the 38 and 50 % sat. $(NH_4)_2SO_4$ procedures (0·35 % flavinphosphate)	—
↓	
After dialysis, adsorb on alumina Cγ and elute twice with $(NH_4)_2HPO_4$ and NH_3. Dialyse. Repeat process (0·53 % flavinphosphate)	—

there should be 2·4 g. of flavoprotein in 100 l. of milk. Since each litre contains 120 g. dry weight, the degree of concentration at the 0·53 % flavinphosphate stage should be 12,000/2·4 = 5000. It is difficult for various reasons to determine directly the initial concentration of flavoprotein in milk. Most important is the fact that fresh milk and the whey powder contain an enzyme which inactivates coenzyme I. Since the presence of flavoprotein at these stages can only be detected by a catalytic test, involving the use of coenzyme I, obviously no accurate estimation of concentration is possible. In addition to this complication, there is the fact that the concentration of flavoprotein in milk lies within the limits of error of the method of detection. The estimation of the loss in the purification process, particularly in the early stages, is approximate and merely gives order of magnitude. Stages 1 and 2 together involve about a 30 % loss mainly due to the bulkiness of the precipitate at 25 % saturation of $(NH_4)_2SO_4$ and *p*H 4·0. Stages 3, 4 and 5 involve losses of approximately 75, 60 and 30 % respectively.

A preparation of flavoprotein at the 0·53 % flavinphosphate stage is not homogeneous in an ultracentrifugal field. The amount of the impurity and the theoretical % of flavinphosphate which a homogeneous preparation should contain are discussed in the appendix by J. St L. Philpot. The low initial concentration of flavoprotein in milk and the great losses in the purification procedure render attempts at further purification extremely expensive. Starting with 50 l. of milk, one practically reaches the vanishing point at the 0·53 % flavinphosphate stage. In the final stages, scarcity of material is the limiting factor in the purification.

II. *Physical and chemical properties*

A concentrated solution of purified milk flavoprotein (> 10 mg./ml.) is orange-red in colour. Dilute solutions (< 2 mg./ml.) appear brownish yellow. On addition of hyposulphite to a neutral solution of the protein, the colour is bleached and restored by shaking with air. Re-oxidation is practically instantaneous as soon as all the hyposulphite is oxidized to sulphite.

Milk flavoprotein is rapidly denatured at room temperature in solutions of *p*H 3·8 or less, likewise in concentrated aqueous solutions (> 40 %) of methyl alcohol and acetone. Temperatures higher than 50° denature the protein within a few min. Denaturation is always accompanied by the liberation of the prosthetic flavin. Indeed the appearance of greenish fluorescence serves as a delicate indicator of denaturation. Loss of catalytic activity can be used as a quantitative measure of denaturation of flavoprotein. No success has yet been attained in the reversible resolution of flavoprotein into prosthetic flavin and undenatured protein.

The absorption spectrum of our purest flavoprotein preparation (0·53 % flavinphosphate) is given in Fig. 1. There are three main absorption bands with maxima at 279, 350 and 450 mμ respectively. The spectrum was recorded by a Hilger "Spekker" photometer. The positions of the band peaks are identical in all preparations examined although with purification the ratio

$$\log \frac{I_0}{I}(279\ \mathrm{m}\mu) \Big/ \log \frac{I_0}{I}(450\ \mathrm{m}\mu)$$

increased.

The spectrophotometric method was used to estimate the flavin content of preparations of known dry weight. The assumption was made that the absorption coefficient β at 450 mμ was $2{\cdot}4 \times 10^7$. The justification for this assumption is that all known flavin-protein compounds have the same β value for their main

absorption band in the visible range of the spectrum. Since $\beta=\frac{1}{c}.\frac{1}{d}.\ln\frac{I_0}{I}$ then by determining the value of $\ln\frac{I_0}{I}$ at 450 mμ in a 2 cm. cell, the concentration of riboflavin, c, in mol. per ml. can be calculated from the formula. The concentration was always expressed in terms of g. % of flavinphosphate.

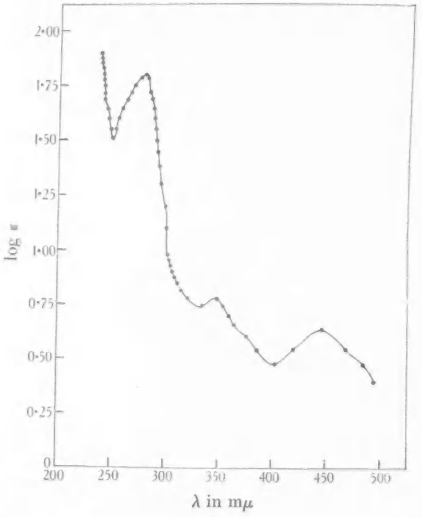

Fig. 1.

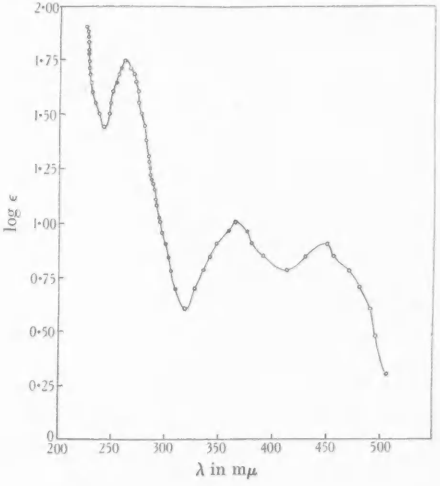

Fig. 2.

Fig. 1. Absorption spectrum of 1·76 % flavoprotein solution at the 0·53 % flavinphosphate stage; *p*H 7.

Fig. 2. Absorption spectrum of the prosthetic flavin after precipitation as the Ag and Ba salts; *p*H 7.

Estimation of the flavin content both directly in the combined form and indirectly after extraction with 75 % methyl alcohol from the protein shows that the yield of free flavin is about 80 %. We are satisfied that this small discrepancy is referable (1) to the inefficiency of extraction, and (2) to the marked tendency of the prosthetic flavin to become adsorbed on the protein precipitate. Fig. 2 shows the absorption spectrum of a purified sample of the prosthetic flavin. There are three main absorption bands with peaks at 260, 365 and 450 mμ. The spectrum is similar to, but not identical with, that of riboflavinphosphate.

III. *Prosthetic flavin*

By extracting a dry preparation of flavoprotein with 75 % methanol, the prosthetic flavin is obtained in solution and the protein is denatured. After removing methyl alchohol by distillation *in vacuo*, and precipitating residual denatured protein with $(NH_4)_2SO_4$, a clear yellow highly fluorescent aqueous solution is obtained. The prosthetic flavin is insoluble in benzyl alcohol; hence it cannot be riboflavin [cf. Emmerie, 1938] and is more probably a phosphorylated derivative. Cataphoretic measurements kindly carried out by Dr R. A. Kekwick of the Lister Institute show that the prosthetic flavin migrates anodically in a

cataphoretic field more rapidly than lactoflavinphosphate. At *p*H 7 in phosphate buffer, at 0° and $\mu = 0{\cdot}02$ the mobilities of lactoflavinphosphate and the prosthetic group were, respectively, $1{\cdot}8 \times 10^{-5}$ and $2{\cdot}4 \times 10^{-5}$ cm.^{2}/volt^{-1}/sec.$^{-1}$. To quote Dr Kekwick: "these measurements are not very accurate owing to the large diffusion constants of the material, but it is safe to say that the unknown flavin migrated the faster". It was therefore clear that the prosthetic group was a phosphorylated derivative not identical with lactoflavinphosphate.

Straub [1938] and Warburg & Christian [1938] have demonstrated that the coenzyme of the amino-acid oxidase is a flavin-adenine dinucleotide. With the assistance of Dr Straub, we have been able to show that the prosthetic group of milk flavoprotein can act as the coenzyme of the amino-acid oxidase (cf. Table II).

Table II

The quantities used were: 0·7 ml. flavin-free amino-acid oxidase prepared by an unpublished method of Dr Straub; 1 ml. phosphate buffer, *p*H 7·2; 0·2 ml. *M* *d*-alanine. Total vol. 2·9 ml. Air in gas space.

Flavin added as μg. flavinphosphate per ml.	0	0·16	0·4	0·8	2
μl. O_2/10 min.	0	36	69	108	108

The high specificity of oxidation coenzymes in general argues the close chemical similarity of the prosthetic flavin and the coenzyme of the amino-acid oxidase, but biological interchangeability is by no means conclusive evidence of identity. In collaboration with A. H. Gordon and S. Williamson we are pursuing further the question of its relation to the amino-acid oxidase coenzyme. The physical and chemical properties of the two compounds do not seem to be identical.

IV. *Catalytic properties*

Reduced coenzyme I (diphosphopyridinenucleotide) is oxidized extremely slowly by methylene blue and other oxidation-reduction indicators. In presence of milk flavoprotein these reactions are enormously accelerated. The catalysis has been quantitatively measured in the following three test systems:

(1) reduced coenzyme + methylene blue;
(2) lactic dehydrogenase system + oxidized coenzyme + methylene blue;
(3) lactic dehydrogenase system + oxidized coenzyme + methylene blue (or pyocyanine) + O_2.

In systems (1) and (2) the velocity of the catalytic reaction is determined by measuring the rate of decoloration of methylene blue under anaerobic conditions. The difference between the two systems lies in the method of reducing the coenzyme. In (1) reduced coenzyme prepared by treating oxidized coenzyme with hyposulphite is used. In (2) the lactic dehydrogenase system is used to reduce oxidized coenzyme *in situ*. Since methylene blue is autoxidizable it is possible to study the reaction manometrically by measuring the rate of oxygen absorption as in (3). Apart from the different methods of measuring the reactions in (2) and (3) respectively, we are dealing in (2) with a steadily falling concentration of methylene blue and in (3) with a constant concentration of methylene blue. There should, therefore, be differences in the respective rates of reaction.

Table III shows the catalytic effect of flavoprotein on the reaction between reduced coenzyme I and methylene blue. The coenzyme was reduced by hyposulphite in $M/2$ $NaHCO_3$ solution according to the method of Green & Dewan [1937]. For the details of the Thunberg technique, cf. Green & Dixon [1934]. The

Table III

The quantities used were: 0·8 ml. of 1 % coenzyme solution and 0·2 ml. 0·0125 *M* methylene blue. Total vol. 2·5 ml.

Flavoprotein as μg. flavinphosphate	0	10	2	1	0·2
Reduction time in min.	15	0·5	0·9	1·5	2·1

rate of reduction of methylene blue by reduced coenzyme is within limits proportional to the concentration of flavoprotein. Increase of the concentration of flavoprotein beyond 4 μg./ml. of flavinphosphate has no influence on the catalytic rate under the conditions of the above experiment. It is also interesting that the efficiency of flavoprotein as a catalyst increases with dilution. In other words, the greatest efficiency is reached when the ratio

$$\frac{\text{concentration of reduced coenzyme}}{\text{concentration of flavoprotein}}$$

becomes very large.

In test system (2), the lactic dehydrogenase+lactate is used as the reducing agent of the coenzyme. The enzyme is prepared from rabbit skeletal muscle by the method of Green *et al.* [1937]. The product of oxidation, pyruvic acid, inhibits the reduction process and must be fixed with cyanide. Fig. 3 shows the dependence of the rate of methylene blue reduction on the concentration of flavoprotein. Above 4 μg. flavinphosphate per ml. increase of flavoprotein concentration effects no further increase in the rate of decoloration of methylene blue. As little as 0·04 μg. flavinphosphate per ml. is sufficient to demonstrate a positive catalytic test. No reduction occurs in absence of flavoprotein.

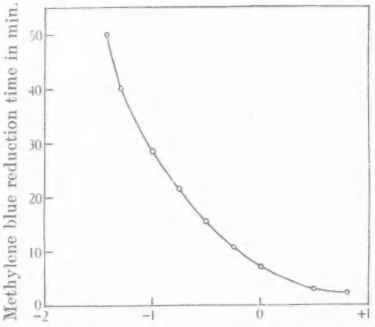

Fig. 3.

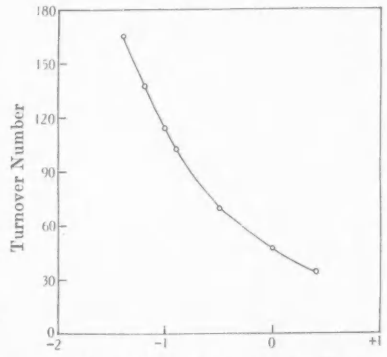

Fig. 4.

Fig. 3. The effect of the concentration of flavoprotein (expressed as μg. flavinphosphate) on the rate of reduction of methylene blue by reduced coenzyme.

Fig. 4. The dependence of the Turnover Number on the concentration of flavoprotein (expressed as μg. flavinphosphate).

Assuming that flavoprotein undergoes a cycle of oxidation and reduction, thereby catalysing the reaction between reduced coenzyme I and methylene blue, one can easily calculate from the above data the number of times flavoprotein would have to be reduced or oxidized in 1 min. (Turnover Number) in

order to account for the overall reaction. The exact molarity of the methylene blue solution was 0·0125. Therefore 0·2 ml. of the methylene blue solution is equivalent to 56 μl. H_2. Also 1 μg. flavinphosphate is equivalent to 0·049 μl. H_2. The Turnover Number (T.N.) is equal to the hydrogen equivalent of the methylene blue reduced per min., divided by the hydrogen equivalent of the flavoprotein present. Fig. 4 shows the dependence of the T.N. on the concentration of flavoprotein. Under the conditions of the experiment a limiting value of about 165 per min. was reached.

In the above experiment, the concentration of coenzyme I was 0·38 mg./ml. The T.N. increases with the concentration of coenzyme. For example with 1 mg. coenzyme, 0·42 μg. flavinphosphate and 0·2 ml. 0·0125 *M* methylene blue in a total volume of 3·6 ml. the T.N. was 105. Repeating the experiment with 3·3 mg. coenzyme the T.N. was 294. Fig. 5 shows the dependence of the T.N. on the concentration of flavoprotein using 2·6 mg. coenzyme per ml. Under optimum conditions, i.e. high coenzyme and low flavoprotein concentrations, the limiting T.N. is about 550.

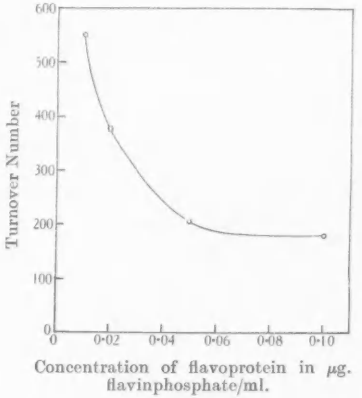

Fig. 5.

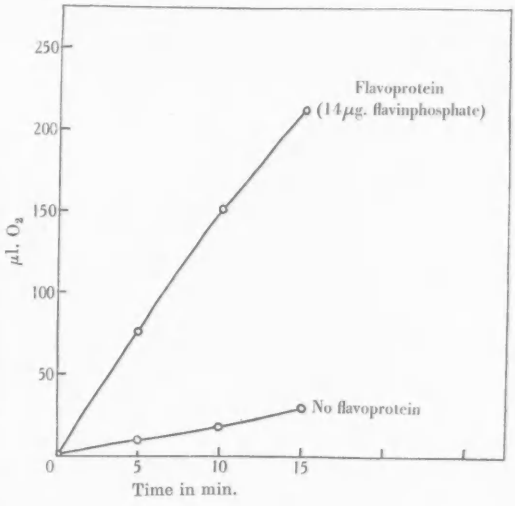

Fig. 6.

Fig. 5. The dependence of the Turnover Number on the concentration of flavoprotein using excess coenzyme. The quantities used were: 1 ml. enzyme; 1 ml. of 0·7% coenzyme I; 0·2 ml. 2*M* HCN; 0·2 ml. 0·0125*M* methylene blue and 0·2 ml. *M* lactate.

Fig. 6. The O_2 uptake of the lactic dehydrogenase system + pyocyanine + coenzyme I ± flavoprotein. The quantities used were: 1·5 ml. enzyme solution; 1 ml. 0·1% coenzyme I; 0·2 ml. 2*M* HCN; 0·2 ml. 0·1% pyocyanine and 0·1 ml. 2*M* lactate.

Table IV

The quantities used were: 1·5 ml. lactic dehydrogenase; 1 ml. 0·1% coenzyme I; 0·2 ml. 2*M* HCN; 0·2 ml. 2*M* lactate; 0·2 ml. 0·1% pyocyanine. Total vol. 4·1 ml.

ml. flavoprotein solution (14 μg. flavinphosphate per ml.)	1·0	0·3	0·1	0·03	0	1·0 (No pyocyanine)
μl. O_2/10 min.	152	94	43	31	22	28

Warburg & Christian [1932] have isolated a flavoprotein compound from yeast which also catalyses the oxidation of reduced coenzyme I by methylene blue. We compared the relative catalytic efficiencies of the two flavoproteins under identical experimental conditions. The ratio of activities milk flavoprotein : yeast flavoprotein was 7. This is in rough agreement with the limiting value of 50 for the T.N. of yeast flavoprotein, as compared with 500 for the milk compound.

The lactic dehydrogenase system + oxidized coenzyme + pyocyanine hardly absorbs oxygen. On addition of flavoprotein a vigorous uptake ensues (cf. Fig. 6). The dependence of the rate of O_2 uptake on the concentration of flavoprotein is shown in Table IV. The blank without flavoprotein is not inappreciable, a fact which rules out experiments with low concentrations of flavoprotein.

There is an unexpected result in Table IV, viz. that in absence of pyocyanine, flavoprotein shows no catalytic activity. In the previous section it was pointed out that reduced flavoprotein is autoxidizable. Two possibilities were open: either that reduced flavoprotein was not autoxidizable under the experimental conditions or that the assumption of flavoprotein undergoing a cycle of reduction and oxidation was incorrect. If the rate of oxidation of reduced flavoprotein by molecular O_2 was the limiting factor we should expect a higher rate of uptake in an atmosphere of O_2 than in air. Experiment showed that flavoprotein in absence of pyocyanine had no catalytic activity, regardless of whether an atmosphere of air or pure O_2 was used. The autoxidation of flavoprotein was therefore not the factor in question. If flavoprotein does not undergo a cycle of reduction and oxidation, it should be possible to demonstrate that flavoprotein is not reduced under the conditions of the experiment. The following experiments show that this is indeed the case. 8 mg. reduced coenzyme and flavoprotein in a concentration of 20 μg. flavinphosphate per ml. were mixed anaerobically in a Thunberg tube at 38°. No decoloration of flavoprotein was observed in the course of 1 hr. In another tube, 0·2 ml. of 0·0125 M methylene blue was added to the above mixture. Reduction of the methylene blue was complete in 30 sec. whereas the colour of oxidized flavoprotein persisted for more than 1 hr. Simple calculation shows that if flavoprotein were undergoing a cycle of reduction and oxidation, it would have to be reduced and oxidized $56/0{\cdot}98 \times 60$ or 3400 times in 1 hr. in order to account for the observed rate of reduction of methylene blue. Yet even in 1 hr. there was no evidence of complete reduction. The same result was obtained when using the lactic dehydrogenase system + oxidized coenzyme instead of hyposulphite-reduced coenzyme. The colour of flavoprotein in these experiments was sufficiently intense to allow visual observation of changes in colour. A control tube with flavoprotein reduced by hyposulphite was used for comparison with the experimental tubes.

The above facts suggest the following picture of flavoprotein catalysis. Reduced coenzyme combines with flavoprotein. The pyridine ring in this complex is easily dehydrogenated by oxidation-reduction indicators such as methylene blue, pyocyanine etc. When oxidation of the pyridine ring takes place, the complex dissociates into oxidized coenzyme and the original flavoprotein. That is to say, flavoprotein remains in the oxidized state during the cycle of its catalysis. The cataphoretic method should easily decide whether such complex formation does in fact take place.

We have carried out similar experiments with the Warburg and Christian flavoprotein of yeast. Although this compound definitely undergoes a cycle of oxidation and reduction our calculations indicate that the cycle is not sufficiently rapid to account for the catalytic effect on the oxidation of reduced coenzyme I

by methylene blue. This may be due to the presence in our yeast preparations of the flavoprotein described by Haas [1938] which does not react directly with O_2.

It is possible to use many variations of the three test systems for demonstrating the catalytic action of milk flavoprotein. For example, the lactic dehydrogenase system can be replaced by other systems which require coenzyme I such as the malic, triose, triosephosphoric and α-glycerophosphoric systems. Similarly methylene blue or pyocyanine can be replaced by flavinphosphate or cytochrome *c*. There were indications that coenzyme I (diphosphopyridinenucleotide) could be replaced by coenzyme II (triphosphopyridinenucleotide), but the experiments were not conclusive.

Preparations of flavoprotein at and beyond the 0·1 % flavinphosphate stage of purity have no xanthine oxidase activity. There is no question, therefore, of the identity of flavoprotein with the xanthine oxidase.

V. *Catalysts for the oxidation of reduced phosphopyridinenucleotides*

There are four catalysts known to catalyse the oxidation of phosphopyridinenucleotides. Table V summarizes essential information about their properties. Coenzyme factor is by far the most active catalyst of this group and is the only

Table V

	Reference	Source	Prosthetic group	Absorption bands mμ	Colour
Coenzyme factor	Dewan & Green [1938], Adler *et al.* [1937]	Animal tissues and yeast	Unknown	—	—
Warburg-Christian flavoprotein	Warburg & Christian [1932], Theorell [1934]	Yeast	Riboflavinphosphate	275, 380, 465	Yellow
Haas flavoprotein	Haas [1938]	Yeast	Dinucleotide of adenine and riboflavin	275, 377, 455	Greenish yellow
Milk flavoprotein	—	Milk	Unidentified flavin	279, 350, 450	Orange-red

one which has a wide distribution in animal tissues and micro-organisms. Nothing is known of its prosthetic group. All three flavoproteins differ in their physical and chemical properties. The differences are referable not only to the flavin but also to the protein portion.

Summary

The isolation and properties of a flavoprotein and its prosthetic group from milk are described.

We are grateful to Messrs Boots, Ltd., and to Mr Slater of this Department for co-operating in the processing of large quantities of milk. One of us (D. E. G.) is indebted to the Ella Sachs Plotz Foundation for a research grant.

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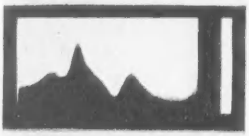
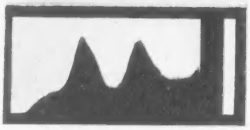
ADDENDUM: EXAMINATION IN THE ULTRACENTRIFUGE

By J. St L. Philpot, *Department of Biochemistry, Oxford* (*with* E. Dodwell, *Technical Assistant*)

Three specimens of milk flavoprotein at different stages of purification were sent by Messrs Corran and Green. The ultracentrifuge results are given in Table I. In every case the flavin appeared to belong wholly to a component having $S_{20} = 11\text{–}12 \times 10^{-13}$. Table I shows that in the purest preparation, containing

Table I

Photograph	Flavin-phosphate %	Sedimentation constants $\times 10^{13}$: Refraction method α	β	γ	δ	Absorption method δ	Concentration of δ component % of total protein
	0·06	1·1	3·9	6·6	11·5	*ca.* 11·0	—
	0·26	—	3·8	6·9	11·7	11·3	15–25
	0·53	—	—	6·2	12·1	12·5	39–46

0·53 % flavin (expressed as lactoflavinphosphate) the flavoprotein forms 39–46 % of the total protein. Hence the pure flavoprotein must contain 1·15–1·36 % flavin, and its mol. wt. divided by the number of flavin groups per mol. must be 34,000–40,000. In this it differs from yeast flavoprotein, which has one flavin group per mol. of weight 78,000 [Kekwick & Pedersen, 1936].

From the sedimentation constant 12×10^{-13} and, assuming the usual partial specific volume 0·75, the minimum mol. wt. (i.e. that of a spherical particle) is about 220,000. Hence there must be at least $220/40 = 5{\cdot}4$ flavin groups per mol. Actually all known proteins with sedimentation constant about 12×10^{-13} have mol. wt. about 280,000 [cf. Svedberg, 1937], which is sixteen times the approximate "Svedberg unit" 17,600. The figure 34,000–40,000 given above suggests that milk flavoprotein may contain one flavin group to every two Svedberg units. If it were composed in all of sixteen Svedberg units it would therefore have eight flavin groups in a molecule of weight 270,000–320,000. Until the homogeneous protein is available this seems the most reasonable assumption.

I am grateful to the Medical Research Council and the Nuffield Trust for financial assistance.

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CCLXXXVIII. STUDIES ON THE KETOGENIC ACTIVITY OF THE ANTERIOR PITUITARY

I. THE RELATION OF KETONAEMIA TO KETONURIA IN THE RAT

II. A METHOD FOR THE ASSAY OF THE KETOGENIC ACTIVITY

III. THE NATURE OF THE KETOGENIC PRINCIPLE

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BURN & LING [1930] were the first to demonstrate that the injection of anterior pituitary extracts into normal fasting rats is followed by an increased urinary excretion of acetone bodies. This observation has been repeatedly confirmed in the rat and a similar increase in the urinary excretion of these substances has also been found in the dog [Rietti, 1934] and guinea pig [Best & Campbell, 1938]. As may be anticipated there also occurs in susceptible animals a simultaneous increase in the blood acetone bodies and this has been found in the rat, dog, man [Anselmino & Hoffmann, 1931] and rabbit [Magistris, 1932; Mirsky, 1936; Best & Campbell, 1936].

The nature of this effect of anterior pituitary extracts has given rise to some differences of opinion. Some investigators have been inclined to attribute it to a separate "ketogenic principle" or fat metabolism hormone [Anselmino & Hoffmann, 1931; 1936; Black *et al.* 1934]. Since the fatty acids represent the chief source of these substances it is not surprising to find that pituitary extracts also cause an increase in liver fat and a decrease in the quantity of depot fat [Anselmino *et al.* 1935; Best & Campbell, 1936; Fry, 1937]. However, as Best & Campbell [1936] have pointed out, wide discrepancies may exist in the magnitude of the ketonuria and the accumulation of liver fat under the influence of these extracts.

While the existence of a separate anterior pituitary principle specifically concerned with the mobilization and metabolism of fatty acids forms an attractive hypothesis for the interpretation of the experimental facts, there are other considerations of the action of anterior pituitary extracts that should be borne in mind before such a view is generally accepted.

The discovery [Houssay & Biasotti, 1931] that hyperglycaemia and glycosuria may be produced in fed dogs by crude anterior lobe extracts has been amply confirmed and since one explanation of their results is that such extracts suppress the utilization of carbohydrate [Russell, 1938], it would not be surprising if under such circumstances an increased proportion of fatty acids was utilized to meet the energy requirements. Furthermore, these crude extracts also produce marked effects on the protein metabolism of normal fed dogs.

[1] National Research Council Fellow in Medicine, 1936–1938.

Teel & Cushing [1930] and Gaebler [1933] have shown that such injections produce not only a fall in the blood non-protein N but also a very striking decrease in the urinary N excretion. Consequently, it would seem that an equally strong case could be made for the view that the occurrence of ketonuria and an increased liver fat content is a secondary effect induced by an anterior pituitary hormone (or hormones) whose main influence is directed upon the carbohydrate and protein metabolism of the organism.

The above difficulty would be resolved if it could be shown that it is possible to isolate from the anterior pituitary gland fractions that independently alter the metabolism of fat, protein and carbohydrate. Anselmino & Hoffmann [1934] have claimed that by selective ultrafiltration at different H^+ concentrations such a separation is possible. They report that one such protein-free fraction will increase the blood acetone bodies without affecting liver glycogen while another causes a decrease in liver glycogen without increasing blood acetone bodies. Magistris [1932] has also reported that the ketogenic principle of anterior pituitary extracts is ultrafilterable. Recently, in a series of preliminary communications, workers in Collip's laboratory have reported that not only is the ketogenic principle ultrafilterable but also that it is heat-stable at *p*H 10 and that they have obtained evidence that this activity is either identical or closely associated with the melanophore-expanding principle of the pituitary [Neufeld & Collip, 1938; O'Donovan & Collip, 1938; Denstedt *et al.* 1938]. Of even greater interest is their statement that this principle will also increase the glycosuria of hypophysectomized and depancreatized dogs, and raise the O_2 consumption and depress the R.Q. in both the normal and thyroidectomized rabbit. Finally, it may be pointed out in this connexion that although both Anselmino & Hoffmann and Magistris report that their ketogenic substance is ultrafilterable they find that the activity is destroyed by heating to 60° for 15 min.

The present paper deals with an attempt to establish more exactly the nature of this ketogenic action of anterior pituitary extracts. Unexpected difficulties were, however, encountered in the development of a reliable assay method for this activity based either upon alterations in the blood or urine ketone bodies, and since these observations have a definite bearing on the main problem they will also be considered at this time.

I. THE RELATION OF KETONAEMIA TO KETONURIA IN THE RAT

The success of any fractionation of the ketogenic activity of anterior pituitary extracts is dependent on the development of an accurate and reproducible method of assay.

The injection of crude saline or alkaline extracts into normal animals is followed by increases in the blood and urine acetone bodies that are mainly dependent on the sex, species and dietary condition of the animal used. Our own experience as well as that of others has indicated that the white rat of 120–150 g. initial weight does not excrete appreciable quantities of acetone bodies over a period of 72 hr. fasting, but the injection of anterior pituitary extracts produces in the majority of animals a well-marked ketonuria. Unfortunately we have observed a great variability in the magnitude of the ketonuria evoked in the responsive animals. Moreover, about 25% of the rats developed no ketonuria,

even though they were drawn from the same stock and were of the same sex as the responsive members.

Such a variability of response, which obviously excludes the induction of ketonuria as a quantitative method of assay, strongly suggests either that certain rats are immune to the extract or else that a definite level of ketonaemia must be reached before ketonuria can be detected. The latter alternative has proved to be the correct one and the following experiments would appear to establish at least in the rat a definite renal threshold for the acetone bodies.

Methods

Male rats weighing 120–150 g. were used exclusively. Determinations of the total acetone-body content of the urine were made by the method of Van Slyke [1917] and of the blood by the method of Van Slyke & Fitz [1917] with slight modifications which permitted the analyses to be made on 3 ml. blood.

The animals were divided into four groups.

(1) The first group of 59 rats had been maintained on a diet of dog chow (26 % protein, 7 % fat, 67 % carbohydrate) before use. They were fasted for 48 hr. and the urinary excretion of acetone bodies was determined during the second 24 hr. of fasting. Following this the animals were exsanguinated under Nembutal anaesthesia and the blood analyses made by a micro-method for the determination of blood acetone bodies which will be described in Part II. A fairly constant level of these substances is reached after some 30 hr. of fasting and this is maintained practically unchanged at least until the 55th hr. Consequently a blood analysis at the end of 48 hr. of fasting should give a fair representation of the level that had prevailed during the preceding 24 hr.

(2) 31 rats which comprised the second group were injected after 24 hr. fasting with 0·5 ml. anterior pituitary extract of proved ketogenic activity and the urinary excretion of acetone bodies during the next 24 hr. was determined. Following this a second injection of 0·5 ml. extract was given and the blood acetone bodies determined 4–5 hr. later.

(3) While exception might be taken to this last procedure on the grounds that the blood samples were not taken during the period of urine collection, we have previously found that the 24-hr. urinary excretion of acetone bodies in a given rat, after the succeeding daily injections of extract, is reasonably similar on the two successive days. However, in order to leave no doubt on this point, in another series of 12 rats we have taken blood during the period of urine collection. The relationship between the blood and urine acetone bodies of these rats was in no way different from those treated as in the first series,

(4) The final small group consisted of 6 rats that had been maintained on a low protein, high fat diet prior to fasting (protein 11 %, fat 40 %, carbohydrate 43 %). Deuel *et al.* [1937] have shown that a diet of this nature markedly increases the ketonuria of rats when they are fasted. In this series the urinary excretion between the 24th and 48th hr. of fasting was determined as well as the blood acetone bodies at the end of this period.

Results

Table I contains the average values and the range of both the urine and blood acetone bodies for these four groups of rats with an additional group of fully fed rats. This table clearly shows that in the control of fasting animals, although the blood acetone bodies varied over a wide range, yet in no animal did the ketonuria exceed 3·8 mg. in the 24 hr. In both series of animals injected with anterior pituitary extracts, although the average blood level was approximately double

that of the controls, the variation in the blood level within both the control and injected groups would make it impossible to decide by a single determination whether or not a given injection had exerted a stimulating action on the formation of acetone bodies. Consideration of the urinary acetone body excretion leads to similar difficulties. Although no control animal excreted more than 4 mg. acetone bodies, yet in one injected group 64 % and in the other 83 % of the rats failed to excrete more than this quantity when injected with 0·5 ml. anterior pituitary extract, a dose which is five times that which will produce ketonuria in some of the animals. It is true that as progressively greater amounts of extract are injected a larger proportion of animals will exhibit ketonuria, but it may be concluded that neither the urinary excretion of ketone bodies nor a *single* determination of blood acetone bodies is an adequate criterion for the presence or absence of ketogenic activity in a given extract unless prohibitively large numbers of animals are used.

Table I

	Treatment	No. of rats	24 hr. excretion of acetone bodies		Blood acetone bodies		% with urine acetone bodies greater than 4 mg./24 hr.
			Mean	Range	Mean	Range	
1	Controls. Fasting 24–48 hr.	59	1·3 ±0·1	0·5– 3·8	12·1 ±1·1	0·8–32·3	0·0
2	Fasting. Injected 0·5 ml. APE	31	8·8 ±2·1	0·5–49·1	23·2 ±1·9	0·5–44·4	36·0
3	Fasting. Injected 0·5 ml. APE	12	7·6 ±4·2	0·3–44·2	23·8 ±4·2	6·0–53·0	17·0
4	Fasting. High fat, low protein diet	6	55·6	35·2–72·7	32·5	25·0–37·6	100·0
5	Fully fed. Dog Chow diet	11	—	—	1·4	0·7– 1·9	—

The reason for the variability in ketonuric response becomes apparent when the blood level of acetone bodies in all four groups of animals is correlated with the urinary excretion (Table II). It will be seen that the urinary acetone body content does not rise above 5 mg./24 hr. until the blood level has reached a value of *c.* 25–30 mg. 100 ml. Beyond this point the excretion rises sharply. The existence of this urinary threshold explains the phenomenon of a complete lack of response in a proportion of fasting rats which have been given anterior pituitary extract. These rats happen to have a low initial level of blood acetone bodies and although the extract produces ketonaemia, the kidney threshold is not exceeded and hence no ketonuria follows the injection.

Table II. *Relation between blood and urine acetone bodies of control and injected rats*

Blood acetone bodies mg. %	No. of animals	Urine acetone bodies mg./24 hr.
0– 4·9	16	1·0 ±0·1
5·0– 9·9	21	1·1 ±0·1
10·0–14·9	8	1·2 ±0·2
15·0–19·9	17	1·9 ±0·4
25·0–29·9	15	6·7 ±2·4
30·0–34·9	9	38·0 ±9·3
35·0	7	28·5 ±6·1

It has previously been shown that acetone itself is not a threshold substance [Widmark, 1920; Briggs & Shaffer, 1921] and consequently this threshold effect

is due either to the behaviour of β-hydroxybutyric acid or to acetoacetic acid or both. The existence of a renal threshold for these substances would be in accord with the view that these compounds are normally utilized by the organism.

Finally, it may be pointed out that the view that the fasting rat does not develop ketosis since it does not exhibit ketonuria would appear to be incorrect inasmuch as the blood level during fasting may exceed that in the fed animal by some twenty-fold (Table I).

II. A METHOD FOR THE ASSAY OF THE KETOGENIC ACTIVITY

The methods used to detect the ketogenic activity of anterior pituitary extracts have hitherto consisted either of determining the ketonuria following their injection into fasted animals, usually rats [Burn & Ling, 1930; 1933; Butts *et al.* 1934; Black *et al.* 1934; Best & Campbell, 1936; Gray, 1938] by the determination of blood ketone bodies in single samples of blood some hours after the injection [Anselmino & Hoffmann, 1931], or by following the blood ketone curve of injected rabbits [Magistris, 1932].

Our results (Part I) indicate that none of these methods will decide with certainty whether or not a given extract possesses activity. Thus, while a large dose of an active extract will unmistakably increase the ketonuria of a fair proportion of animals yet smaller doses of the same extract will fail to indicate the presence of the active principle. On the other hand, a single determination on the blood would be of value were it not for the extreme individual variation in the fasting ketonaemia, in consequence of which a large number of determinations must be made on both fasting and injected animals before statistically significant conclusions can be drawn (Table I).

The most desirable method, without question, would be one in which the acetone body content of the blood could be determined on the same animal both before and at a given time after injection. However, since the reliable blood acetone methods which exist at present require the use of fairly large blood samples for each determination, only animals of sufficient size to yield a copious quantity of blood can be successfully used. Magistris [1932] has employed rabbits in this manner for the assay of ketogenic activity, but it has been our experience with the strains of rabbits at our command that even large doses of an active extract often fail to produce any marked increase in ketonaemia even though the animals are first fasted for 48 hr. Dingemanse [1936] has likewise had no success in producing ketonaemia by the injection of anterior pituitary extract in this species.

Since the fasting young rat is perhaps the animal most sensitive to this action of anterior pituitary extract it appeared necessary to devise a micro-method by which serial determinations could be made of the acetone body content in the quantities of blood that might be withdrawn from the tail (*c.* 0·2 ml.). The reagent described by Scott-Wilson [1911] is sufficiently sensitive to be used for the nephelometric determination of the acetone body content in blood samples of this magnitude provided that the oxidation and distillation for the conversion of acetoacetic acid and β-hydroxybutyric acid into acetone can be performed with reasonable uniformity and accuracy. This reagent has already been used in this manner for the determination of acetone bodies in larger quantities of blood by Marriott [1913] and Rietti [1937].

The method of Abels [1937] is adaptable to 0·2 ml. blood but is only applicable to the determination of acetone and acetoacetic acid which comprise not more than 20–30 % of the total acetone bodies.

The following method for the assay of the ketogenic activity of anterior pituitary extracts was evolved and has proved the only procedure which, in our hands, has yielded consistently satisfactory results.

Procedure

Male rats weighing between 120 and 150 g. which had been maintained on a stock diet of Purina Dog Chow before being used, were fasted for 48 hr.; 24 hr. is not sufficient because the ketonaemia does not reach a plateau until the 30th hour after withdrawal of food. We have not found the fed rat to be sufficiently sensitive to pituitary extract to use it for the assay.

An initial sample of blood is obtained as will be described below. The extract under examination is then injected intraperitoneally, and a second sample of blood withdrawn 4–5 hr. later. Although the rise in blood acetone bodies after the injection of an active preparation does not reach a maximum for about 8 hr., it is sufficiently near its peak within 4 hr. for the purposes of assay. A response has been considered positive only when the blood acetone body content has increased 4 mg./100 ml. or more above the control level.

It has also been observed that rats which have an initial fasting level of less than *c.* 3 mg./100 ml. are often relatively unresponsive and therefore rats which had a fasting ketonaemia of 2·5 mg./100 ml. or less were not included in any series for assay.

Determination of total blood acetone bodies in 0·2 ml. blood

Blood was obtained by section of the tip of the tail after preliminary exposure of the rat to the heat of an electric bulb. The blood was gently milked from the tail on to a watch glass coated with K oxalate, 0·2 ml. blood taken up into an accurately calibrated pipette and transferred at once to a 15 ml. graduated centrifuge tube containing 10 ml. $N/70$ H_2SO_4. After stirring with a fine rod and allowing to stand for 10 min., 0·2 ml. 10 % Na tungstate solution was added and the mixture again well stirred and allowed to stand for an additional 2–3 min. It was then centrifuged and all the clear supernatant fluid poured into the distilling flask (*A*) of the apparatus shown in Fig. 1. This apparatus was made entirely of pyrex glass with the glass joint carefully ground in order to eliminate the use of grease, the presence of which is not desirable. The volume of the precipitate adherent to the centrifuge tube was noted (0·8–1·0 ml.) and subtracted from the total volume in order to obtain the true quantity of filtrate analysed.

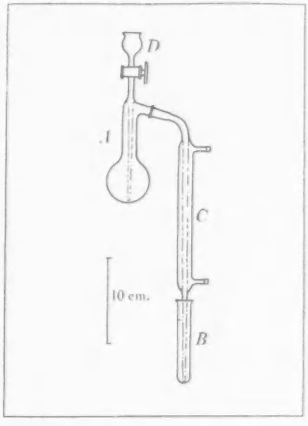


Fig. 1.

Two ml. 2 % $NaHSO_3$ were placed in the receiving tube (*B*) of the apparatus and the latter placed beneath the condenser (*C*) so that the outlet tube dipped almost to the bottom of the receiving tube.

The distilling flask was then heated with the flame of a shielded micro-burner. When the contents just reached the boiling point, the flame was slightly lowered

and 2 ml. 2 % K dichromate in 20 % H_2SO_4 to which a little talc powder had been added were run through the stopcock (*D*) which was then closed and the flame adjusted so that 5 ml. were distilled over into the receiving tube in the course of 20–30 min. It is advisable to calibrate the receiving tube so that distillation may be stopped when this volume has been collected. The contents of the receiving tube (7 ml.) were transferred to a 50 ml. Erlenmeyer flask and 5 ml. Scott-Wilson reagent added while the flask was whirled to insure uniform and rapid mixing. The receiving tube was then washed out with the mixture. Any delay or lack of thoroughness in the mixing will lead to erroneous and irregular results. Subsequent agitation should be avoided.

A series of standards for comparison with the unknown solution was prepared by placing 2 ml. $NaHSO_3$ in the flasks, then adding the requisite amount of a standard acetone solution and enough water to bring the volume to 7 ml. The Scott-Wilson reagent (5 ml.) was then added as above. A stock solution containing 1 mg. acetone per ml. may be kept in a closely stoppered bottle in the refrigerator for months without serious deterioration. One ml. of this solution was diluted to 50 ml. just before use; 1 ml. diluted solution is then equivalent to 10 mg./100 ml. of blood acetone. Standards equivalent to 5, 10 and 20 mg./100 ml. will suffice.

The unknown and the standard solutions are kept for 15 min. and then compared either in a colorimeter, using a blue filter, or else in a nephelometer.

The time of standing before comparison is of some importance since the turbidity develops rather slowly when quantities of less than 5 mg./100 ml. acetone are present. It reaches a maximum after approximately 20 min. with these low concentrations while, on the other hand, with concentrations above 5 mg./100 ml. the maximum turbidity is reached within 3–4 min. In the determination at high concentrations a period of standing beyond 5 min. causes some aggregation and consequently a slight diminution in turbidity; however, this error is not of serious magnitude below values of 25 mg./100 ml.

The method is sensitive to blood acetone body concentrations as low as 1·5–2·0 mg./100 ml. and, if desired, the preformed acetone and acetoacetic acid may be determined separately. To determine these 5 ml. 0·25 % H_2SO_4 are added to the blood filtrate in the distilling flask and a preliminary distillation carried out before the addition of potassium dichromate.

Results

In Table III are recorded a series of consecutive determinations of acetone and β-hydroxybutyric acid. Of the former 20 μg. were added to 12 ml. water; of the latter, sufficient was added to whole blood to give initial concentrations of 5, 10 and 20 mg./100 ml. in terms of acetone.

Table III. *Recoveries of acetone and of β-hydroxybutyric acid*

20 μg. acetone in 12 ml. water. % recovery: 85, 87, 81, 92, 82, 90, 90, 84, 82, 85. Average 85 %

β-Hydroxybutyric acid expressed as acetone in blood sample mg./100 ml.	Converted and recovered as acetone %	Average %
5	77, 63, 73, 76	79
10	73, 65, 70, 75	71
20	72, 64, 64, 65	66

The average recovery of acetone is 85 %; while the conversion of β-hydroxybutyric acid into acetone averages 65–70 %, a value that compares favourably

with that obtained by other methods. The variability in the results is, in actual practice, of little consequence since the significant blood changes that are measured are always of a much greater order of magnitude. A combination of aeration and distillation will raise the recovery of acetone alone to 95%, but leads to a still greater variability in the quantity of β-hydroxybutyric acid converted into acetone even when the rate of air flow is carefully controlled.

It was discovered that simple boiling of a very dilute solution of acetone (20 μg. in 10 ml.) for 15 min. in a sealed glass container resulted in a 15% loss. The low recovery of acetone by distillation is thereby explained. Aeration undoubtedly allows a higher recovery by virtue of the fact that the acetone is rapidly "washed out" at a temperature somewhat below 100°. In the determination of total acetone bodies a reduction in temperature during aeration would undesirably influence the oxidation of β-hydroxybutyric acid and thereby reduce the yield of acetone.

The use of this method to detect the ketogenic activity of small quantities of anterior pituitary extract is illustrated in Fig. 2. The activity of such quantities of extract would be difficult, if not impossible, to detect if reliance had to be

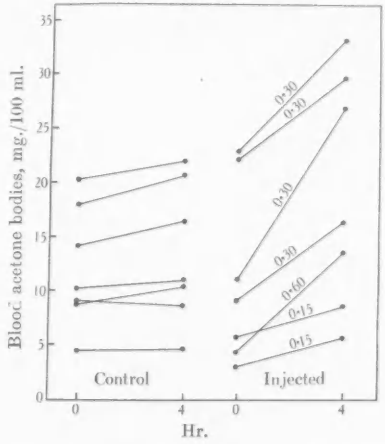


Fig. 2. The effect of anterior pituitary extract on the blood acetone bodies of fasted rats. The quantity in mg. of organic solids injected is indicated on the curves.

placed on single blood estimations or on the ketonuria over a period of 24 hr. A most gratifying aspect of the use of the method has been the relative constancy with which various animals show either a positive or negative response to small doses of a given extract.

III. THE NATURE OF THE KETOGENIC PRINCIPLE

The development of a sensitive method for detecting the ketogenic activity of anterior pituitary extracts (Part II) has enabled us to investigate certain claims that have been advanced as to the chemical nature of the substance responsible for this activity.

(*a*) *Heat-stability.* All workers with the exception of Neufeld & Collip [1938] have found the ketogenic principle to be heat-labile. Our results show that heating previously active extracts in the boiling water bath at *p*H 10 for 10 min. destroys their activity (Table IV).

Table IV. *Effect of heating at* p*H 10 in boiling water bath for 10 min. on the ketogenic activity of anterior pituitary extracts*

Extract	Amount injected mg. protein	Blood acetone bodies (mg./100 ml.) 0 hr.	4 hr.
A, unheated	1·4	7·2	14·2
,,	1·4	6·6	11·0
heated	1·4	7·2	8·1
,,	1·4	2·8	3·0
B, unheated	0·3	9·8	16·9
,,	0·3	11·0	15·5
heated	2·0	10·4	10·1
,,	2·0	3·6	5·0

(*b*) *Ultrafiltration.* Anselmino & Hoffmann [1931; 1936], Magistris [1932], and more recently Denstedt *et al.* [1938], have reported that the ketogenic principle will pass through membranes that retain proteins, i.e. cellophane and 8% acetic-acid-collodion. In our experience, however, this principle will not pass through such membranes. Our starting materials have been both aqueous extracts of acetone-dried glands and alkaline extracts of fresh glands. The filtration was carried out under suction at *p*H 8·0–9·0, and both the original ultrafiltrate and that concentrated fourfold *in vacuo* failed to influence the blood acetone body content of the fasted rat (Table V). However, the residue remaining in the sacs still possessed marked activity in much smaller doses than those given of the ultrafiltrate. The latter, although free from protein, still contained considerable quantities of N (0·2–0·25 mg. per ml.).

It is well known that membranes may be made with any desired pore size; those that we have used would neither permit proteins nor the substance responsible for the ketogenic activity of the anterior pituitary to pass. It would be necessary to employ a series of graded membranes in order to determine the

Table V. *Effects of* (*A*) *original extract,* (*B*) *its ultrafiltrate, and* (*C*) *a boiled extract of pituitary "colloid" on the blood acetone bodies of fasted rats*

Extract	Amount injected ml.	mg. N	Melanophore expanding activity frog units/ml.	Blood acetone bodies mg./100 ml. 0 hr.	4 hr.	% change
(A) Original	0·03	0·04	1,300	22·0	29·4	+ 34
,,	0·03	0·04	1,300	22·8	32·9	+ 44
,,	0·03	0·04	1,300	11·0	26·7	+143
,,	0·03	0·04	1,300	9·0	16·2	+ 80
(B) Ultrafiltrate	1·5	0·35	20	13·5	13·4	± 0
	1·5	0·35	20	8·4	7·8	− 8
Concentrated ultrafiltrate	1·5	1·4	200	22·9	24·1	+ 5
	1·5	1·4	200	8·8	7·0	− 20
(C) Pituitary "colloid"	1·0	—	10,000	14·9	17·0	+ 14
,,	1·0	—	10,000	4·0	2·5	− 38
,,	2·0	—	10,000	7·6	6·9	− 9
,,	2·0	—	10,000	3·9	3·7	− 5
,,	2·0	—	10,000	10·6	9·7	− 9
,,	2·0	—	10,000	9·3	11·0	+ 16

molecular size of this substance. Our findings suggest, however, that the molecule is a relatively large one.

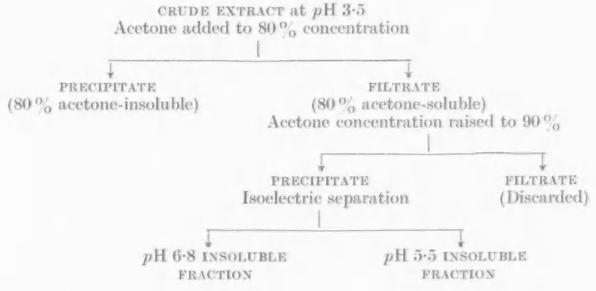
(*c*) *The melanophore-expanding principle.* In view of the suggestion by O'Donovan & Collip [1938] and Denstedt *et al.* [1938] that the ketogenic activity is either identical or closely associated with the melanophore-expanding principle, we have prepared from 10 g. beef pituitary "colloid", by extraction with boiling 0·25% acetic acid, an extract that contained 10,000 frog units per ml. of this principle. This extract, in doses of 1–2 ml., did not increase the blood acetone bodies of fasted rats (Table V) nor did it increase the glycosuria of partially depancreatized rats when administered in doses as large as 20,000 units, although these same rats responded to doses of crude anterior pituitary extract containing only 1000 units of melanophore principle.

The heat-lability of the ketogenic principle (Table IV) also speaks against its identity with the melanophore principle since the activity of the latter was somewhat increased by heating at *p*H 10 for 15 min. It may be mentioned that crude anterior pituitary extracts also lost their glycosuric activity in the partially depancreatized rat after heating, a confirmation of the report of Houssay & Biassotti [1931].

(*d*) *Fractionation of the protein of the anterior pituitary.* Inasmuch as the present evidence would indicate that the hormones of the anterior pituitary are either proteins or else closely associated with proteins, we have made various attempts to concentrate the ketogenic activity by simple protein fractionations. Neither isoelectric precipitation of alkaline or saline extracts, nor fractional precipitation by different concentrations of ammonium sulphate gave any clear-cut separation of active from inert material, since the ketogenic activity appeared to spread itself over a number of different fractions.

The following plan of fractionation was the most satisfactory in the sense that a fair degree of separation of the various pituitary principles was achieved and that a ketogenic fraction was obtained relatively free from certain of the other hormones (Table VI).

Table VI



Four vol. acetone are added to a Burn & Ling extract [Burn & Ling, 1933] after bringing the *p*H to 3·5 with dil. HCl. If flocculation does not occur, a small amount of sat. NaCl is stirred in until there is a distinct separation of flocculent precipitate from clear solution. After centrifuging and filtering through hardened filter paper, the clear light brown filtrate is treated with an equal vol. acetone and allowed to stand about $\frac{1}{2}$ hr. The precipitate is centrifuged off, dissolved in a vol. of water equal to $\frac{1}{4}$ of the original extract, and the *p*H brought to 6·8. The

isoelectric precipitate is taken up in water containing enough NaOH to bring to *p*H 10–11. Dil. HCl is then added until the *p*H reaches 7·5–8·0. There should be a distinct turbidity at this point, but no flocculation. The solution is then frozen, thawed and the greenish-gray precipitate centrifuged off; dissolved in alkali again and refrozen at *p*H 7·5–8·0, as before. For the final solution this precipitate is dissolved in alkali at *p*H 9·0, or acid at *p*H 5·5. The three supernatants from the above precipitations are pooled, the *p*H lowered to 5·5; and the precipitate, which may be enhanced in yield by freezing, dissolved in water and alkali and brought to *p*H 7·0. The final solutions may be preserved indefinitely if kept frozen at $-10°$ to $-20°$.

The acetone-insoluble fraction and the two acetone-soluble fractions were assayed for the familiar anterior lobe hormones in order to determine how the ketogenic potency was affected by the fractionation as compared with the other hormonal activities. The results are summarized in Table VII.

The methods which were used for assay were as follows: 100 g. hypophysectomized rats, 14–20 days after operation, were used for growth assay. The intracutaneous crop test of Lyons & Page [1935] was employed for assaying the lactogenic principle. The chick method of Smelser [1937] has proved both simple and reliable for the assay of thyrotropic activity. 21-day old male rats were used for adrenotropic assays [Moon, 1937] and gonadotropic activity was determined by the mouse uterus method as used by Levin & Tyndale [1937]. The familiar darkening of the bleached skin of the frog was utilized for the detection of melanophore activity. Ketogenic assays were performed as already described. The partially depancreatized rat has proved to be an excellent test object for the "diabetogenic" principle. Many of these rats do not exhibit a spontaneous glycosuria, but will readily respond after the injection of anterior pituitary extract. Although quantitative assays of the "diabetogenic" principle were not attempted, these animals serve as very satisfactory test objects to establish the presence or absence of the substance which is capable of producing hyperglycaemia and glycosuria. For the sake of convenience the various potencies are expressed

Table VII. *Relative hormonal potencies*

(Units per mg of organic solids.)

	Thyrotropic	Ketogenic	Diabetogenic	Growth	Lactogenic	Adrenotropic	Gonadotropic	Melanophore
Original Burn & Ling extract	10–15	4–8	Active	15–25	100	10 mg. produced 25 % increase in wt. of adrenals	—	1300
80 % acetone-insoluble fraction	30	—	—	—	—	—	—	—
80 % acetone-soluble *p*H 5·5 fraction	None*	4–8	Active	15–25	1000	—	—	—
80 % acetone-soluble *p*H 6·8 fraction	None*	4–8	Active	15–25	10	10 mg. produced 21 % increase in wt. of adrenals	None†	5

* No response with 3·5 mg. † No response with 8·5 mg.

in terms of arbitrary units wherever possible. A unit refers simply to the minimal dose in terms of organic solids which produces an unquestionable response in the majority of test animals. With the thyrotropic, growth and adrenotropic assays units are expressed in terms of daily dose, otherwise the total dose is referred to.

The outcome of the series of assays was significant (Table VII) not only because of the fact that ketogenic and "diabetogenic" activities were seen to run parallel with each other, but also because their occurrence coincided with that of the growth principle. It had appeared from preliminary assays that the *p*H 5·5 fraction would be low in growth-promoting activity, but in the end it became quite clear that this fraction was fully as potent as any other.

The *p*H 6·8 isoelectric fraction, in addition to being free from thyrotropic principle, was also quite low in lactogenic principle, and contained no detectable gonadotropic activity. A moderate amount of adrenotropic activity was present and the very low content of melanophore principle in this fraction again speaks against this hormone being responsible for the ketogenic and "diabetogenic" effects of anterior pituitary extracts.

The assays are disappointing in the fact that they reveal that there has been no concentration of ketogenic activity as the result of the process of fractionation. While this may be due to a destruction of some of the active material during the fractionation procedure, the retention of ketogenic, growth and "diabetogenic" activity in equal amounts is a fact of some significance. Furthermore, the absence of all or most of the thyrotropic, gonadotropic, lactogenic and melanophore principles from the *p*H 6·8 fraction allows us to conclude that these hormones are not of chief importance in the production of these effects on metabolism.

Additional information as to the identity of the ketogenic principle is obtained by the assay of the two proteins obtained by the Lyons [1937] acid acetone extraction method for the preparation of prolactin (Table VIII).

Table VIII. *Relative hormonal potencies—Lyons acid acetone fractions*

	Lactogenic	Adrenotropic	Ketogenic	Glycosuric	Growth
*p*H 5·5 fraction	10,000 units per mg.	—	Less than $\frac{1}{4}$ unit per mg.	No response with 20 mg.	Less than $\frac{1}{4}$ unit per mg.
*p*H 6·5 fraction	—	10 mg. produced 29% increase in wt. of adrenals	No response with 7 mg.	No response with 17 mg.	No response with 4 mg. per day

The *p*H 5·5 fraction has an exceedingly high content of lactogenic activity, while the *p*H 6·5 fraction, although not especially rich in adrenotropic activity, at least contained more of this principle than has been found in other preparations. Both these fractions, however, contained, at the most, only traces of growth, ketogenic and "diabetogenic" activities as judged by our methods of assay.

Discussion

The practice of assuming that the multiple effects of anterior pituitary extracts upon metabolism are due to separate hormones does not seem desirable in the absence of any chemical evidence that this is the case. On the contrary, a consideration of the known facts of metabolism indicates that some of the alterations produced by the injection of anterior pituitary extracts may be regarded as secondary consequences of their action. Among these we suggest the production of ketosis may be included.

Our reasons for this assumption are not only our failure to confirm the claim of Anselmino and Hoffman that ultrafiltration will yield a product that specifically increases the acetone body content of the blood and urine, but are based on the contention that the well-substantiated action of anterior pituitary extracts on the carbohydrate and protein metabolism must of necessity increase the proportion of fat undergoing catabolism and, in consequence, increase the

formation of acetone bodies. Thus, it has been shown that the injection of these extracts into fed normal or hypophysectomized rats results in a decreased utilization of carbohydrate as evidenced by a fall in R.Q., an increase in blood sugar and in muscle and liver glycogen [Fisher *et al.* 1936; Meyer *et al.* 1937; Russell, 1938]. There is little reason to suspect that a similar action of anterior pituitary extracts in suppressing carbohydrate utilization does not occur in fasted rats, even though the utilization of carbohydrate under these conditions is reduced to a low level. Now, since it is in the fasted rat that the ketogenic action of this extract is most readily demonstrated, it may be suggested that this is a necessary consequence of its effect on carbohydrate utilization and is not due to a specific stimulation of fatty acid catabolism. Although it might be argued that a further reduction in the already low level of carbohydrate metabolism would not account for the magnitude of the observed ketosis it is necessary to point out that anterior pituitary extracts also decrease the proportion of protein undergoing catabolism. This is shown by the fall in blood non-protein N and urinary N excretion that follows their injection into fed animals [Teel & Cushing, 1930; Gaebler, 1933]. In addition, Lee & Schaffer [1934] have found that under these circumstances there is an increased retention of protein in the body. This effect has been attributed to the growth-promoting principle of the pituitary and is not associated with either the thyrotropic or gonadotropic principles [Gaebler, 1935, 1; 1938]. Furthermore, in the fed dog, Gaebler [1935, 2] has conclusively shown that this protein-sparing effect is accompanied by an increased utilization of fat. In the fasting rat, Harrison & Long [1938] have observed a 30–40 % reduction in urine N on treatment with anterior pituitary extracts rich in growth-promoting, "diabetogenic" and ketogenic activities. Consequently, although in the fasted rat a further reduction in carbohydrate utilization may be of little consequence, the removal of such a large proportion of protein from catabolism must increase the proportion of fatty acid catabolism and offers a satisfactory explanation for the ketosis and fatty infiltration of the liver that follows these injections.

This view of the mode of action of the anterior pituitary removes the necessity for postulating a hormone specifically concerned with fat metabolism and most certainly for a separate principle whose function is to increase the acetone body content of the blood and urine. Such a view is also in accord with our present inability to dissociate by chemical means the ketogenic activity of the anterior pituitary from its growth-promoting and "diabetogenic" activities. Even if the latter two effects are ultimately shown to be due to separate hormones it does not lessen the present argument that a decreased utilization of carbohydrate or protein must be reflected by an increased catabolism of fatty acids since the energy requirements of the organism must still be met.

Since, in the fasting animal, the sole source of carbohydrates is the tissue protein, this effect of the anterior pituitary in decreasing protein catabolism may be regarded as an advantage to the organism. In a larger sense there must exist some critical mechanism which determines whether a cell will store protein or catabolize that already present. In such a mechanism a hormonal factor, derived from the anterior pituitary, might be of primary importance.

Summary

1. The existence of a urinary threshold for acetone bodies in the rat renders the development of ketonuria in response to anterior pituitary extracts in this animal unsuitable for purposes of assay. The increase in blood acetone-bodies will not give rise to ketonuria unless this urinary threshold is exceeded.

2. While the fed rat shows a negligible increase in blood acetone bodies in response to the injection of anterior pituitary preparations, the fasting rat responds quite readily. Fasting alone however produces a distinct but exceedingly variable ketonaemia. In consequence, single blood analyses made on injected rats are not reliable for the determination of ketogenic activity unless extremely large groups of animals are used.

3. A micro blood acetone body method is described which employs only 0·2 ml. tail blood, and thus allows a control blood acetone reading to be made on a given animal before injection of an extract. This method makes it possible to detect the presence of a small quantity of ketogenic principle and to determine the minimal effective dose.

4. The ketogenic principle could not be filtered through 8% acetic collodion or cellophane. It was destroyed by heating on the boiling water bath for 15 min. at *p*H 10.

5. Comparative assays of relative potencies of the various anterior pituitary hormones present in a number of different preparations obtained by chemical fractionation, indicated that ketogenic activity followed growth and "diabetogenic" activity. This finding suggests that a single principle may be responsible for all of these three effects of the anterior pituitary on metabolism.

6. It is pointed out that the inhibitory action of anterior pituitary extracts on the catabolism of carbohydrate and protein offers a satisfactory explanation for the occurrence of the ketosis that follows their injection.

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CCLXXXIX. OBSERVATIONS ON TISSUE GLYCOLYSIS[1]

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The substances whose action will be described are mainly: (1) the ammonium ion, (2) glutamic acid and (3) maleic acid. They affect tissue glycolysis in different ways: either, as with (1), by raising the aerobic glycolysis and at the same time depressing anaerobic glycolysis, or, as with (2), mainly by inhibiting anaerobic glycolysis, or, as with (3), by a slow and continuous rise of the aerobic glycolysis to the normal level of the anaerobic glycolysis. The observed effects only occur or are specially pronounced in brain tissue.

Methods

The experiments were carried out at 37·5° in the Warburg apparatus. Tissue slices, suspended in bicarbonate or phosphate Krebs-Ringer solution containing 0·2 % glucose (*p*H 7·4), were used. In most experiments respiration and glycolysis were simultaneously measured by Warburg's differential method [1930]. Additions to the suspension medium were present from the beginning in most cases, so that the first reading was taken 15–20 min. after the tissue came in contact with the solution. For brain, embryo and medulla of kidney R.Q. of 1, for testis, intestinal mucosa and spleen R.Q. = 0·9 and for Jensen sarcoma R.Q. = 0·8 were assumed [cf. Dickens & Šimer, 1930; 1931; Dickens & Weil-Malherbe, 1936].

Estimations of lactic acid were made according to Friedemann & Kendall [1929] after copper-lime treatment, estimations of NH_3 by steam distillation *in vacuo* and subsequent Nesslerization according to Parnas & Heller [1924].

I. The effect of ammonium ions

Ashford & Dixon [1935] found that addition of $M/10$ KCl to the Ringer solution increases both respiration and aerobic glycolysis of rabbit brain slices, while inhibiting their anaerobic glycolysis. Later, Dixon & Holmes [1935] and Dickens & Greville [1935] showed that the same effect can be obtained with CsCl and RbCl. The effect is absent from tumour, kidney, testis and yolk sac of the rat [Dickens & Greville, 1935] and from chick embryo [Needham *et al.* 1937]. According to Dixon [1937] the effect on brain tissue is seen equally well with $M/20$ KCl, though it is only small at $M/100$.

NH_4Cl affects respiration and glycolysis of brain tissue in a very similar way: $M/30$ NH_4Cl causes a rise of respiration as well as of aerobic glycolysis up to the

[1] Most of the experimental work was carried out in spring and summer 1935, partly at the Biochemical Laboratory, Cambridge. The results were reported at the meeting of the Biochemical Society, December 1935 [see Weil-Malherbe, 1935].

normal level of the anaerobic glycolysis, while at the same time inhibiting the anaerobic glycolysis. As with KCl this effect is, with few exceptions, restricted to brain tissue (Table II). In spleen an increased aerobic glycolysis was found. An effect comparable with that in brain was also observed with intestinal mucous membrane in one experiment. But in another experiment with the same tissue this effect was very small and in a third experiment inhibition of both respiration and glycolysis occurred. The figures for the metabolism of the control were very similar to each other in these and other experiments.[1] With several other glycolysing tissues inhibition of anaerobic glycolysis by $M/30$ NH_4Cl was observed, but the effect on respiration and aerobic glycolysis was negligible or, at most, a slight suggestion of that found with brain.

The action of NH_4^+ on brain however differs in one respect from those of K^+, Cs^+ or Rb^+. Whereas in these cases concentrations $> M/100$ are apparently necessary to produce an effect, the effect of NH_4^+ is still recognizable at concentrations as small as $0{\cdot}3 \times 10^{-3}$ M (Table I), which still cause a marked increase of aerobic glycolysis and also a small stimulation of respiration. Low concentrations ($M/1000$ and less) sometimes seem to stimulate the anaerobic glycolysis as well; but in other experiments, reported in Table XI, inhibition was still observable at $M/1000$.

Table I. *The effect of NH_4^+ on brain metabolism. Slices of guinea-pig brain (grey matter) in bicarbonate-saline*

	Concentration 10^{-3} M NH_4Cl	Q_{O_2} 1st hr.	Q_{O_2} 2nd hr.	$Q_G^{O_2}$ 1st hr.	$Q_G^{O_2}$ 2nd hr.	$Q_G^{N_2}$ 1st hr.
(1)	33·3	−19·5	−10·2	19·2	12·1	4·4
	10	−11·6	− 9·0	8·0	6·1	6·0
	3·3	−11·8	− 8·6	11·2	8·8	19·6
	1	−10·6	− 8·7	9·2	7·2	26·0
	0	− 6·9	− 5·8	3·1	0·6	20·4
(2)	1	−10·8	− 8·8	10·2	7·6	16·0
	0·33	− 9·3	− 6·3	6·8	2·5	15·9
	0·1	− 7·0	− 6·3	4·0	1·0	17·8
	0	− 6·8	− 6·0	4·4	0·9	16·8

Dickens & Greville [1935] explained the K^+ effect as the result of a disturbance of the ionic balance of the medium leading to physical changes in the colloidal structure of the cell protoplasm and probably to increased permeability. The same may be true for NH_4^+ in high concentration, but with NH_4^+-concentrations of the order of 10^{-3} M a specific toxic effect seems more likely.

II. The effect of glutamic acid

(*a*) *Action on the anaerobic glycolysis*

During his studies on glutamine formation in nervous tissues Krebs [1935] discovered the inhibition of anaerobic lactic acid formation by glutamic acid. On his suggestion I investigated the phenomenon in greater detail.

l(+)Glutamic acid, in a concentration of $M/100$, inhibits the anaerobic glycolysis of brain slices by 30–70%. The inhibition increases with time and

[1] The figures reported here for the normal metabolism of rat intestinal mucous membrane differ from those found in the literature [e.g. Rosenthal & Lasnitzki, 1928]. We observed usually a high aerobic glycolysis of the same order as the respiration. Both activities showed a rather rapid fall.

Table II. *Metabolism of various glycolysing tissues with and without M/30 NH_4Cl. Bicarbonate-glucose-Ringer*

		With NH_4Cl						Control					
		Q_{O_2}		$Q_G^{O_2}$		$Q_G^{N_2}$		Q_{O_2}		$Q_G^{O_2}$		$Q_G^{N_2}$	
Species	Tissue	1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.
Guinea-pig	Medulla of kidney	−10·2	− 9·7	14·0	12·8	21·6	21·5	− 6·8	− 6·9	14·3	13·1	24·0	22·0
Guinea-pig	Retina	—	—	—	—	61·6	—	—	—	—	—	51·7	—
Rat	Testis	−11·3	− 5·1	3·7	2·1	6·0	4·9	− 7·5	− 7·9	2·9	1·8	7·4	6·5
Rat	Spleen (*a*)	−17·5	—	6·8	—	9·0	—	−10·0	—	4·4	—	11·7	—
	(*b*)	−15·2	−15·4	4·5	7·7	5·0	5·4	−12·9	−12·7	4·2	4·2	7·8	7·3
Rat	Diaphragm	—	—	—	—	3·8	—	—	—	—	—	4·1	—
Rat	Jensen sarcoma	− 8·2	−14·7	25·6	19·2	30·9	24·2	−14·4	−12·4	22·8	21·9	29·0	21·6
Mouse	Yolk sac*	—	—	—	—	14·8	10·6	—	—	—	—	24·6	22·4
Mouse	Embryo†	− 8·9	− 8·1	2·9	1·7	9·5	7·7	−10·4	− 8·2	3·1	3·0	7·1	6·2
		1–10 min.	1–60 min.	1–10 min.	1–60 min.	1–10 min.	1–60 min.	1–10 min.	1–60 min.	1–10 min.	1–60 min.	1–10 min.	1–60 min.
Rat	Intestinal mucosa (*a*)	−47·5	−25·0	59·5	33·6	22·1	14·4	−18·7	−16·4	42·0	24·2	45·6	25·0
	(*b*)	−26·7	−18·1	28·4	18·5	23·2	14·8	−24·0	−15·4	26·8	15·5	38·4	20·4
	(*c*)	− 6·3	− 3·7	12·9	6·3	—	13·2	−27·3	−13·4	32·2	15·8	—	20·5

* Almost full term. † Average dry wt. 8 mg.

reaches its maximum value after about 60 min. (Table III). Variation of the glutamic acid concentration between $M/50$ and $M/1000$ does not greatly affect the inhibition.

Table III. *Inhibition of anaerobic glycolysis of brain slices by l(+)glutamic acid*

		$Q_{G}^{N_2}$						
		With glutamic acid		Control		Inhibition %		
Species	Conc.	1–10 min.	60–80 min.	1–10 min.	60–80 min.	1–10 min.	60–80 min.	Remarks
Rat	$M/100$	10·5	5·1	16·0	13·8	35	63	Mean of 4 exp.
Guinea-pig	$M/100$	12·2	6·0	21·0	18·4	42	67	Mean of 8 exp.
Rat	$M/1000$	13·5	5·4	15·6	12·1	13·5	55·5	
Guinea-pig	$M/1000$	18·2	8·5	18·9	14·8	4	42·5	Mean of 4 exp.

The effect seems to be specific for brain (Table IV). Even in retina it was found to be absent or, at the most, very feeble. Neither the anaerobic glycolysis of yeast cells, nor that of defibrinated blood (guinea-pig), nor the acid production from starch in a dialysed muscle extract (rabbit) was inhibited by glutamic acid.

Table IV. *Effect of l(+)glutamic acid on the anaerobic glycolysis of various glycolysing tissues*

		$Q_{G}^{N_2}$ (1st hr.)	
Species	Tissue	With $M/100$ glutamate	Control
Guinea-pig	Retina	48·7	51·8
Pigeon	Retina	76·0	81·0
Guinea-pig	Medulla of kidney	27·6	29·0
Rat	Intestinal mucosa	16·3	15·5
Guinea-pig	Intestine (whole wall)	7·5	6·9
Guinea-pig	Spleen	5·3	5·9
Rat	Testis	7·2	7·5
Rat	Diaphragm	6·1	5·9
Rat	Jensen sarcoma	36·9	39·2
Rat	Yolk sac	13·1	12·9
Guinea-pig	Heart	7·2	6·5

Specificity of the effect. A few closely related substances cause similar inhibition of the anaerobic glycolysis of brain tissue. They are: (1) the non-natural isomeride, $d(-)$glutamic acid; (2) glutamine; (3) *dl*-β-hydroxyglutamic acid[1] (Table V and VI). $d(-)$Glutamic acid is slightly less efficient than $l(+)$glutamic acid: at a concentration of $M/3000$ where the *l*-acid is still seen to cause some inhibition even a stimulating effect appears. The effects of the two stereoisomerides in different concentrations are compared in the same experiment (Table V). The actions of glutamine and hydroxyglutamic acid are shown in Table VI. Oxidized and reduced glutathione are also included, although they did not affect the anaerobic glycolysis; there was perhaps an initial activation by the reduced glutathione. The observation of Geiger [1935] that oxidized glutathione inhibits the anaerobic glycolysis of chopped brain could not be confirmed with brain slices. Geiger's results have been severely criticized by Dixon [1937].

Apart from the substances mentioned no other amino-acid of 13 tested[2] ($l(+)$alanine, $l(+)$valine, $l(-)$leucine, $l(-)$methionine, $l(-)$proline, $l(-)$hydroxy-

[1] Gift from Prof. Harington to Dr Krebs.

[2] Concentrations $M/100$. Retention was allowed for where necessary.

Table V. *Inhibition of anaerobic brain glycolysis by various concentrations of* l(+)- *and* d(−)*glutamic acids*

	With *l*(+)glutamic acid					With *d*(−)glutamic acid				
		$Q_G^{N_2}$		% Inhibition			$Q_G^{N_2}$		% Inhibition	
Species	Conc. 10^{-3} *M*	1–10 min.	60–80 min.	1–10 min.	60–80 min.	Conc. 10^{-3} *M*	1–10 min.	60–80 min.	1–10 min.	60–80 min.
Rat	0	15·6	12·1	—	—	0	15·6	12·1	—	—
	10	9·7	3·6	38	70	10	13·2	5·9	15	51
	3	10·9	3·9	30	68	3	11·9	5·8	23·5	52
	1	13·5	5·4	13·5	55	1	13·6	6·9	13	43
	0·3	16·9	9·4	0	22	0·3	20·1	12·7	0	0
Guinea-pig	0	19·6	15·4	—	—	0	19·6	15·4	—	—
	10	14·5	6·7	26	56·5	10	16·3	7·2	17	53
	3	15·2	7·6	22·5	51	3	16·9	11·5	14	25
	1	16·8	6·0	14	61	1	22·6	12·5	0	19
	0·3	17·0	12·2	13	21	0·3	23·5	18·9	0	0

Table VI. *Specificity of the inhibition of anaerobic brain glycolysis by glutamic acid and related substances*

		$Q_G^{N_2}$		% Inhibition		
Species	Addition	1–10 min.	60–80 min.	1–10 min.	60–80 min.	Remarks
Rat	0	16·7	14·8	—	—	Mean of 2 exp.
	M/100 *d*(−)glutamic acid	12·8	8·8	23	41	
Guinea-pig	0	21·1	17·9	—	—	Mean of 5 exp.
	M/100 *d*(−)glutamic acid	16·6	8·5	21	53	
Guinea-pig	0	19·3	17·7	—	—	
	M/100 *l*(+)glutamic acid	10·5	5·5	45	69	
	M/100 *dl*-β-hydroxyglutamic acid	13·6	6·8	30	62	
	M/100 glutamine	14·8	8·1	23	54	
	M/500 glutamine	18·8	14·1	3	20	
Rabbit	0	17·5	12·8	—	—	
	M/100 *d*(−)glutamic acid	12·0	7·1	31	45	
	M/100 glutamine	10·1	4·8	42	62	
Guinea-pig	0	18·6	17·0	—	—	
	M/100 glutathione (GSSG)	18·1	16·8	—	—	
Guinea-pig	0	14·0	10·1	—	—	Gas mixture freed from O_2 over hot copper
	M/100 glutathione (GSH)	19·7	11·8	—	—	

proline, *dl*-serine, *l*(−)aspartic acid, *l*(−)pyrrolidonecarboxylic acid, *l*(+)ornithine, *l*(+)arginine, *l*(−)histidine, *l*(−)tryptophan) affected the anaerobic glycolysis of brain slices. In addition a large number of mono-, di- and tri-basic organic acids (for some of them see Table XVI) were tested. Except for a slight unspecific inhibition in a few cases, no effect was found. The effect of the glutamic acid group is therefore quite specific.

(*b*) *Augmentation of the glutamic acid effect on anaerobic glycolysis*

Meyerhof & Lohmann [1926, 1, 2] found that lactic acid in *M*/100–*M*/50 concentration inhibited anaerobic glycolysis of rat brain by about 50%. As in the case of glutamic acid they observed a similar effect of the natural and unnatural isomerides. Dickens & Greville [1933] who reinvestigated this effect found a similar inhibition of anaerobic brain glycolysis only with higher concentrations (*M*/10–*M*/20); they also found *d*(−)lactate less efficient than *l*(+)lactate.

$M/100$ *dl*-lactate did not inhibit the anaerobic glycolysis of guinea-pig brain in our experiments; in an experiment with rat brain however some inhibition was observed. $M/100$ lactate however always enhanced the inhibition caused by glutamic acid. This effect is especially large, when small concentrations of glutamic acid are used (Table VII).

Table VII. *Increase of the glutamic acid effect on anaerobic brain glycolysis*

		$Q_{O_2}^{N_2}$		% inhibition		
Species	Addition	1–10 min.	60–80 min.	1–10 min.	60–80 min.	Remarks
Guinea-pig	0	19·9	17·5	—	—	
	$M/100$ *dl*-lactate	18·9	16·3	5	7	
	$M/100$ *l*(+)glutamate	13·1	7·3	34	58	
	$M/100$ *dl*-lactate + $M/100$ *l*(+)glutamate	9·6	3·7	52	79	
Rat	0	18·3	10·3	—	—	
	$M/100$ *dl*-lactate	14·4	6·2	21	40	
	$M/1000$ *l*(+)glutamate	12·4	6·6	32	36	
	$M/100$ *dl*-lactate + $M/1000$ *l*(+)glutamate	7·8	3·1	57	70	
Guinea-pig	0	23·1	20·0	—	—	Mean of 4 exp.
	$M/100$ succinate	22·5	19·1	3	4	
	$M/100$ *l*(+)glutamate	14·8	7·0	36	65	
	$M/100$ succinate + $M/100$ *l*(+)glutamate	8·3	3·5	64	82·5	
Guinea-pig	0	24·2	21·2	—	—	
	$M/1600$ *l*(+)glutamate	21·6	14·7	11	31	
	$M/100$ succinate + $M/1600$ *l*(+)glutamate	19·6	9·6	19	55	
		1st hr.	2nd hr.	1st hr.	2nd hr.	
Guinea-pig	0	17·4	17·1	—	—	
	$M/100$ *dl*-lactate	19·8	16·3	0	5	
	$M/100$ succinate	16·8	15·6	3	9	
	$M/100$ *l*(+)glutamate	9·1	4·9	48	71	
	$M/100$ *dl*-lactate + $M/100$ *l*(+)glutamate	7·9	4·2	55	75·5	
	$M/100$ succinate + $M/100$ *l*(+)glutamate	7·2	2·6	59	85	

Another substance which unexpectedly increased the glutamic acid effect was found to be succinic acid. $M/100$ succinic acid alone never affected the anaerobic glycolysis. In combination with glutamic acid however the residue of the anaerobic glycolysis observed in presence of glutamic acid alone was again reduced by nearly 50 %. The effect can again best be demonstrated with small concentrations of glutamic acid (Table VII, Fig. 1).

Many other substances which were tried in combination with glutamic acid did not influence its action.

(c) *Reversibility of the inhibition of anaerobic glycolysis*

The glutamic acid inhibition of anaerobic glycolysis of brain slices can be reversed by pyruvic acid (Table VIII, Fig. 2). Variation of the pyruvic acid concentration between $M/100$ and $M/1000$ does not change the result. The fall of anaerobic glycolysis is not entirely prevented, but only delayed. After some time inhibition again prevails. Pyruvic acid also relieves the inhibition caused by *d*(−)glutamic acid and by the combination of glutamic + succinic acids. The

action of pyruvic acid is less efficient if it is added after the first 20 min. of the experiment.

A similar effect, though to a smaller extent, is displayed by α-ketoglutaric acid (Table VIII).

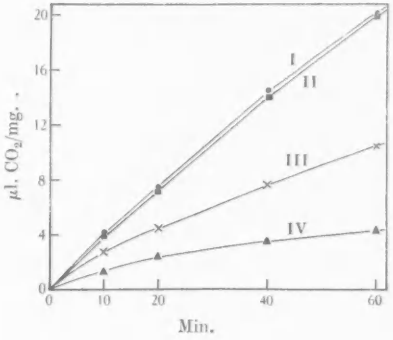

Fig. 1.

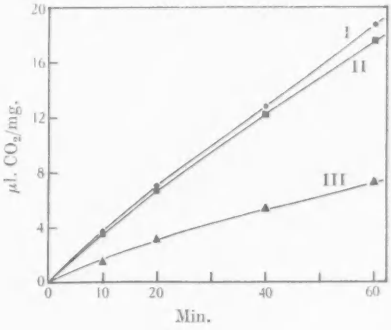

Fig. 2.

Fig. 1. Anaerobic glycolysis of guinea-pig brain slices in bicarbonate-glucose-Ringer. I, no addition; II, with $M/100$ succinate; III, with $M/100$ $l(+)$glutamate; IV, with $M/100$ succinate + $M/100$ $l(+)$glutamate.

Fig. 2. Anaerobic glycolysis of rat brain slices in bicarbonate-glucose-Ringer. I, with $M/100$ pyruvate; II, with $M/100$ pyruvate + $M/100$ $l(+)$glutamate; III, with $M/100$ $l(+)$glutamate.

Table VIII. *Reversal of the glutamic acid effect on anaerobic brain glycolysis*

Species	Addition	$Q_{O_2}^{N_2}$ 1–10 min.	$Q_{O_2}^{N_2}$ 60–80 min.	% Inhibition 1–10 min.	% Inhibition 60–80 min.
Rat	0	16·7	14·9	—	—
	$M/500$ pyruvate	21·7	17·8	—	—
	$M/100$ $l(+)$glutamate	9·1	6·0	45·5	60
	$M/100$ $l(+)$glutamate + $M/500$ pyruvate	21·6	15·3	0	0
Guinea-pig	0	23·2	19·4	—	—
	$M/100$ pyruvate	22·8	20·3	—	—
	$M/100$ $l(+)$glutamate	15·3	8·4	34·0	56·7
	$M/100$ $l(+)$glutamate + $M/100$ pyruvate	22·0	13·6	5	30
Guinea-pig	0	21·2	18·7	—	—
	$M/100$ $l(+)$glutamate + $M/100$ succinate	10·3	4·9	51·5	74
	$M/100$ $l(+)$glutamate + $M/100$ succinate + $M/100$ pyruvate	19·6	13·7	7·5	26·6
Guinea-pig	0	20·6	17·7	—	—
	$M/100$ $l(+)$glutamate + $M/100$ succinate + $M/100$ pyruvate	17·5	13·4	15	24
	$M/100$ $l(+)$glutamate + $M/100$ succinate + $M/300$ pyruvate	16·7	11·9	19	34
	$M/100$ $l(+)$glutamate + $M/100$ succinate + $M/1000$ pyruvate	19·8	15·2	4	14
Guinea-pig	0	16·9	11·9	—	—
	$M/100$ $d(-)$glutamate	16·0	7·8	5·5	34·5
	$M/100$ $d(-)$glutamate + $M/100$ pyruvate	21·0	10·9	0	8
Guinea-pig	0	18·7	17·0	—	—
	$M/100$ α-ketoglutarate	23·2	21·0	—	—
	$M/100$ $l(+)$glutamate	14·3	7·4	23·5	56·5
	$M/100$ α-ketoglutarate + $M/100$ $l(+)$glutamate	18·2	11·4	3	33

As Mendel *et al.* [1931] have shown, pyruvic acid also counteracts the inhibition of anaerobic glycolysis of tumour tissue caused by glyceraldehyde. The same is true for embryonic tissue [Needham & Nowiński, 1937]. In brain however pyruvic acid cannot prevent the inhibition of anaerobic glycolysis by glyceraldehyde [Holmes, 1934; Baker, 1938].

The increased CO_2 evolution in presence of pyruvic acid is not due to an increased decarboxylation of pyruvic acid. The rate of anaerobic disappearance of pyruvic acid was determined by the carboxylase method after incubation with brain slices in presence and in absence of glucose and of *l*(+)glutamic acid. It was found to be independent of these (Table IX). A similar result has recently

Table IX. *Anaerobic disappearance of pyruvic acid after incubation with guinea-pig brain slices*

Initial concentration of pyruvic acid: *M*/200. Duration of exp.: 90 min.

Addition	$Q_{pyruvate}$ Exp. 1	$Q_{pyruvate}$ Exp. 2
0	−3·8	−3·5
0·2% glucose	−4·4	−3·0
0·2% glucose + *M*/100 *l*(+)glutamate	−4·3	−3·2

been reported by Kritzmann [1938] who found that the disappearance of pyruvic acid from minced brain is not increased by the presence of *l*(+)glutamic acid. From a Q-value = 3–4 for pyruvic acid disappearance an extra CO_2 production of 1·5–2 (Q_{CO_2}) can be deduced [cf. Weil-Malherbe, 1937]. This is too small to account for the restoration of the acid production after inhibition by glutamic acid.

(*d*) *Mechanism of the glutamic acid effect*

(1) Since it is known that brain tissue can convert *l*(+)glutamic acid into α-ketoglutaric acid by oxidative deamination [Weil-Malherbe, 1936] the described effects might have been due to a liberation of NH_3 which indeed inhibits the anaerobic glycolysis of brain in fairly low concentrations, as has been shown in the first section of this paper. There are however several objections to this view: (*a*) the deamination is an oxidative process and does not occur anaerobically, at any rate not to any significant extent. (*b*) During the deamination of *l*(+)glutamic acid, owing to secondary reactions, the concentration of free NH_3 in the suspension medium does not rise. This has been well established for brain tissue under aerobic conditions [Krebs, 1935; Weil-Malherbe, 1936]. Table X

Table X. *Anaerobic ammonia formation by guinea-pig brain slices*

Addition (0·2% glucose in all exp.)	$Q_G^{N_2}$ 1st hr.	$Q_{NH_3}^{N_2}$ 1st hr.
0	17·3	0·15
M/100 *l*(+)glutamate	11·4	0·11
M/100 *d*(−)glutamate	11·4	0·23
M/100 pyruvate	15·9	0·18
M/100 *l*(+)glutamate + *M*/100 pyruvate	15·9	0·21
M/100 *d*(−)glutamate + *M*/100 pyruvate	14·3	0·23

shows that there is no increase of free NH_3 in presence of *l*(+)- or *d*(−)glutamic acid under anaerobic conditions either. (*c*) *M*/1000 *l*(+)glutamic acid sometimes has a greater inhibitory effect than *M*/1000 NH_4Cl (Table XI).

Table XI. *Comparison of the inhibitions of anaerobic glycolysis of guinea-pig brain slices by* M/*1000* l(+)*glutamate and* M/*1000* NH_4Cl

Addition	$Q_G^{N_2}$ (1st hr.) Exp. 1	Exp. 2	Exp. 3
0	13·3	15·4	16·8
M/1000 *l*(+)glutamate	10·2	9·5	13·7
M/1000 NH_4Cl	9·3	9·5	16·0

In spite of these facts it could be argued that a transitory liberation of a very small amount of NH_3 inside the cell may suffice to produce the same effects as a higher concentration in the surrounding medium. However, though a very slight deamination of *l*(+)glutamic acid cannot be excluded, this explanation cannot account for the effects of *d*(−)glutamic and β-hydroxyglutamic acids which are, as far as we know, not deaminated by brain tissue. The sample of *d*(−)glutamic acid used was probably optically pure. It was a gift from Dr Krebs and was prepared from the racemic acid by yeast fermentation. Analytical figures were published by Krebs [1935].

(2) Braunstein & Kritzmann [1937] suggested that the intermolecular transfer of amino groups, discovered by them, might, in presence of glutamic acid, lead to the formation of alanine instead of lactic acid from glucose and that this process might explain the inhibition of lactic acid formation by glutamic acid. This reaction would however involve the formation of an equivalent amount of α-ketoglutaric acid from glutamic acid, so that the rate of acid formation would remain the same and no inhibition would result in manometric experiments. It is of course possible that the "trapping" of the pyruvic acid arising during glycolysis deprives the glycolytic enzyme system of its natural hydrogen acceptor and that its replacement by the ketoglutaric acid formed from glutamic acid does not restore the original activity. The reversing effect of added pyruvate supports such an interpretation. But against it the following facts must be considered: (1) there is no increased consumption of pyruvate in presence of glutamic acid, at least as far as can be concluded from carboxylase estimations, (2) since *d*(−)glutamic and β-hydroxyglutamic acids act similarly to *l*(+)glutamic acid, one must assume that they can replace it in the process of amino transfer.

(3) *l*(+)Glutamic, *d*(−)glutamic and *dl*-β-hydroxyglutamic acids were the only substances found by Krebs [1935] to react with the enzyme concerned with the synthesis and hydrolysis of glutamine, the first as a natural substrate, the latter two causing competitive inhibition. Though there is as yet no other evidence for it, it is not impossible that the state of this enzyme, whether combined or free, has something to do with the control of glycolysis in nervous tissues. This would explain why the glutamic acid effect is specific for nervous tissue which is the only one of the highly glycolysing tissues where a synthesis of glutamine occurs.

(*e*) *Action on aerobic glycolysis*

Whereas the substances of the glutamic acid group depress the glycolysis of brain tissue under anaerobic conditions, they provoke on the other hand an appreciable aerobic lactic acid formation. Freshly cut brain slices, suspended in bicarbonate-glucose-Ringer solution have a fairly high aerobic glycolysis during the first 10–20 min. of the experiment ($Q_G^{O_2}=4$–6). Normally this aerobic glycolysis soon disappears entirely in the further course of the experiment. In the presence of the substances of the glutamic acid group (i.e. *l*(+)- and *d*(−)glutamic acids, glutamine and *dl*-β-hydroxyglutamic acid) it remains at

this high level. The respiration is unchanged or even accelerated, especially with *l*(+)glutamic acid. Reduced glutathione which differs from these substances in its action on anaerobic glycolysis has the same effect upon aerobic glycolysis of brain slices [see also Baker, 1937]. I confirmed Baker's finding that the autoxidation of reduced glutathione becomes quite negligible after a short incubation with brain slices. Nevertheless, only the results of the chemical lactic acid analysis are reproduced in Table XII.

Table XII. *Effect of glutamic acids on aerobic glycolysis of guinea-pig brain slices*

Addition	Q_{O_2} 1st hr.	$Q_G^{O_2}$ 1–10 min.	$Q_G^{O_2}$ 60–80 min.	Remarks
0	−10·3	3·7	1·0	Mean of 6 exp.
M/100 *l*(+)glutamate	−13·7	5·6	4·8	
0	−10·7	3·8	1·3	Mean of 4 exp.
M/100 *d*(−)glutamate	− 9·2	8·7	6·3	
0	− 9·9	4·5	1·7	
M/100 *l*-glutamine	−13·8	7·3	6·1	
0	−10·3	3·0	0·6	
M/100 *dl*-β-hydroxyglutamate	−10·1	5·2	4·1	

	$Q_G^{O_2}$ (2 hr.) Manometrically	Chemically
0	0·8	1·9
M/100 *l*(+)glutamate	4·6	5·5
M/100 *d*(−)glutamate	5·2	6·0
M/100 glutathione (GSH)	—	5·3

The rise of aerobic glycolysis is usually greater with the non-natural than with the natural glutamic acid.

In no other glycolysing tissue was an increase of aerobic glycolysis observed in presence of *l*(+)glutamic acid (Table XIII).

Table XIII. *Aerobic metabolism of various glycolysing tissues with glutamic acid*

Species	Tissue	With *M*/100 *l*(+)glutamate Q_{O_2}	With *M*/100 *l*(+)glutamate $Q_G^{O_2}$	Control Q_{O_2}	Control $Q_G^{O_2}$
Rat	Testis	− 9·9	3·1	− 7·5	3·8
	Spleen	−10·4	2·8	− 9·3	2·6
	Intestine (whole wall)	− 8·0	4·6	− 7·7	4·7
	Jensen sarcoma (*a*)	−10·5	23·3	− 9·7	20·9
	(*b*)	−12·7	26·1	−12·3	30·8
	Yolk sac	− 4·4	1·9	− 2·5	3·9
Guinea-pig	Medulla of kidney	−10·7	14·2	−11·3	13·8

Pasteur-Meyerhof quotient: respiration, aerobic and anaerobic glycolyses of brain slices were simultaneously determined in several cases with both *l*(+)- and *d*(−)glutamic acids. Owing to the inhibition of the anaerobic glycolysis the aerobic glycolysis almost reaches the level of the anaerobic glycolysis. Thus the Pasteur-Meyerhof quotient $\left(\text{P.M.Q.} = \frac{Q_G^{N_2} - Q_G^{O_2}}{Q_{O_2}}\right)$ which was 1·8–2·0 in the controls fell to 0·2–0·6 in presence of glutamic acid. This is usually interpreted as an

indication that the Pasteur reaction is inhibited. But it is doubtful whether much significance can be attributed to these figures. We do not know whether or not the reactions which lead to an inhibition of glycolysis under anaerobic conditions also operate under aerobic conditions. Indeed, in the presence of high concentrations of NH_4^+ or K^+, the aerobic glycolysis rises to the level of the normal anaerobic glycolysis, while the anaerobic glycolysis is strongly inhibited in the same medium. This is obviously a case where aerobic and anaerobic conditions cannot be compared and where the P.M.Q. becomes meaningless. The same may be true for the effects of glutamic acid. As long as the aerobic glycolysis does not reach the level of the *normal* anaerobic glycolysis it is not safe to assume a breakdown of the Pasteur mechanism though, as in this case, the P.M.Q. may be practically zero.

III. The effect of maleic acid

Preliminary notes

(1) In several experiments solutions of maleic acid were prepared from freshly distilled maleic anhydride. The results differed in no way from those obtained with a sample of commercial maleic acid.

(2) Since the effects observed often change so quickly with time, the usual method of calculating average figures for respiration and glycolysis over periods of hours does not give an adequate impression of the real phenomena. The Q-values were therefore calculated for periods of 20 min. and the figures plotted at the midpoint of the corresponding period. Of course only a limited proportion of the experiments which were actually done can thus be communicated.

It will be noticed that $Q_{O_2}^{1/3}$ often assumes negative values in the control experiment after some time. This is probably due to oxidative disappearance of acid initially formed.

(3) *Retention*: Solutions of $M/50$ maleic acid in bicarbonate-Ringer at pH 7·4 have a retention of about 9 %. In the early experiments no allowance for this was made, since it was believed that the smallness of the retention could not greatly influence the results and did not warrant the rather complicated extra analyses and calculations involved. Later however the retention was allowed for, using a new simplified principle developed by Dickens [unpublished]. It appeared that, if no allowance for retention were made, the curve of the aerobic glycolysis was quite similar to, and only slightly lower than the true glycolysis, corresponding to the proportion of the retention. The respiration, too, was hardly affected as long as the aerobic glycolysis was low. When the aerobic glycolysis was high, however, the errors became very serious indeed the respiration appearing too high by 100–150 %. Thus a secondary rise of respiration following the rise of aerobic glycolysis was revealed as spurious. With $M/50$ mixtures of glutamic and maleic acids or citric and maleic acids which have a retention of 12–14 % the errors amounted to several hundred per cent.

(a) The effect on brain metabolism

The action of maleic acid as a respiratory poison is well known [Thunberg, 1920; Grönvall, 1924; Gözsy & Szent-Györgyi, 1934]. I find that the inhibition of brain respiration depends (1) on the substrate present, (2) on the medium used: it is higher in phosphate than in bicarbonate medium.

(1) The inhibition in phosphate-Ringer solution is largest with glucose as substrate and only slightly less with lactate. It is on the other hand comparatively small with succinate and pyruvate, though it increases with time (Table XIV).

Table XIV. *Inhibition of brain respiration (guinea-pig) in phosphate-Ringer by M/50 maleate in presence of various substrates*

	Q_{O_2}						% Inhibition		
	Without maleate			With maleate					
Substrate added	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.
0·2% glucose	− 9·8	−10·0	−10·3	− 4·3	−1·2	−0·8	56	88	92
M/50 lactate	−10·9	−10·7	−10·8	− 6·3	−1·6	−0·8	42	85	92
M/50 pyruvate	− 9·9	− 9·3	− 9·3	− 8·9	−5·9	−3·5	10	36	62
M/50 ketoglutarate	− 9·2	− 7·9	− 7·0	− 7·2	−3·0	−1·3	22	62	81
M/50 succinate	−12·0	−10·5	− 8·3	−11·3	−6·9	−4·2	6	34	49
M/50 fumarate	− 9·1	− 7·1	− 4·6	− 7·5	−3·3	−1·5	18	54	78

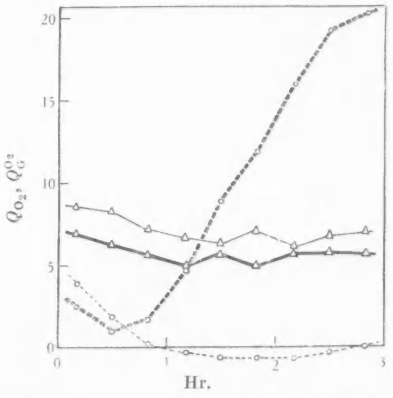

Fig. 3. Respiration and aerobic glycolysis of guinea-pig brain slices. Thick line: with *M*/50 maleate. Retention allowed for. Thin line: control in bicarbonate-glucose-Ringer. △——△ respiration. o- - -o aerobic glycolysis.

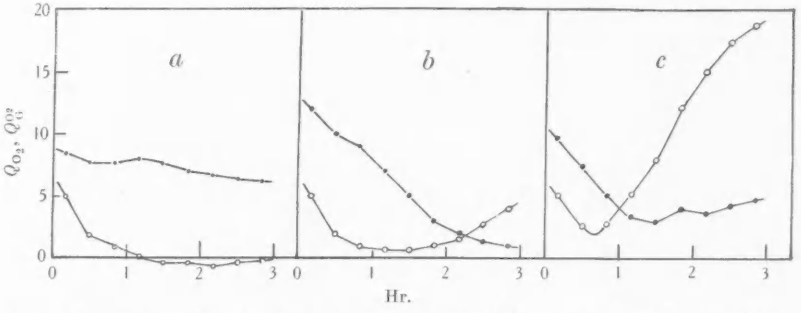

Fig. 4. Respiration and aerobic glycolysis of guinea-pig brain slices. (*a*) control; (*b*) with *M*/100 maleate; (*c*) with *M*/50 maleate. Retention allowed for. •——• respiration. o——o aerobic glycolysis.

(2) In bicarbonate-Ringer solution with glucose as substrate the following observations were made: in presence of *M*/100 maleate there is a slow and steady fall of respiration which at the end of the 3rd hr. reaches almost zero. The aerobic glycolysis does not differ much from the control; a slow rise is sometimes

observed in the 3rd hr. (Fig. 4). If the maleate concentration is increased to $M/50$, the fall of respiration is much less severe. In one experiment (Fig. 4) respiration fell to about 40–50% of the control within the first 20 min. and remained at this level. In another experiment (Fig. 3) there was only about 20–30% inhibition throughout. On the other hand there was a steady rise of the aerobic glycolysis starting after 30–60 min. and reaching the level of the normal anaerobic glycolysis towards the end of the 3rd hr. Values of $Q_{G}^{O} = 24$ have been observed (see Fig. 7). It seems that the onset of the strong aerobic glycolysis prevents the complete breakdown of respiration in presence of $M/50$ maleate. A concentration of $M/100$ maleate is too small to bring about an early and sufficiently large rise of aerobic glycolysis and therefore the paradoxical result is obtained that in this case the respiration is finally inhibited more strongly than with $M/50$ maleate.

In absence of glucose no acid formation occurs. Chemical analysis shows that the acid formed from glucose is lactic acid (Table XV).

Table XV. *Lactic acid formation (by chemical estimation) in presence of maleate. Guinea-pig brain*

Addition	Q_{lactate} mean of 3 hr.
0	2·5
$M/100$ maleate	5·3
$M/100$ maleate + $M/100$ glutamate	8·2
$M/100$ maleate + $M/100$ glutathione	9·6
0	2·7
$M/50$ maleate	7·2
$M/50$ maleate + $M/50$ glutamate	7·8
$M/50$ maleate + $M/50$ ketoglutarate	6·8

Specificity of the maleic acid effect. Though the action of maleic acid is not entirely a specific inhibition of the Pasteur reaction, it is of considerable interest for several reasons: (1) the inhibition of respiration is comparatively small; (2) maleic acid is a simple organic compound closely related to normal metabolites of the cell. Although there are several di- and tri-basic acids which are known to have toxic actions on cell metabolism, none of these caused such a drastic increase of aerobic glycolysis. The following acids have been tested: malonic, hydroxymalonic, hydroxymaleic, tartaric, racemic, dihydroxytartaric, oxalic, itaconic and citraconic acids (all in $M/50$ concentration). Oxalic acid, for instance, inhibited both respiration and glycolysis. Others, like hydroxymalonic acid, had an inhibitory action on brain respiration which was certainly not smaller than that of maleic acid. But although there was no inhibition of the anaerobic glycolysis,[1] the aerobic glycolysis was not significantly increased. $M/50$ maleic acid did not inhibit the anaerobic glycolysis of brain slices (Table XVI). This is in contrast to the experiments of Morgan & Friedmann [1938, 2] who, it is true, worked under different conditions since they used a concentration of $M/12{\cdot}5$ and minced brain suspended in phosphate buffer.

It may be mentioned in this connexion that $M/50$ malonate does not affect the respiration of brain slices in bicarbonate-glucose-Ringer solution. In phosphate-glucose-Ringer there is an inhibition of 40–50% (Table XVI).

[1] Jowett & Quastel [1937] found an inhibition of anaerobic brain glycolysis with $M/14$ hydroxymalonate.

Table XVI. *Metabolism of guinea-pig brain slices in presence of dibasic acids (non-metabolites)*

B = Bicarbonate-glucose-Ringer; P = phosphate-glucose-Ringer.

Addition	Medium	Q_{O_2} 1st hr.	Q_{O_2} 2nd hr.	Q_{O_2} 3rd hr.	$Q_{O}^{O_2}$ 1st hr.	$Q_{O}^{O_2}$ 2nd hr.	$Q_{O}^{O_2}$ 3rd hr.	$Q_{O}^{N_2}$ 1st hr.	$Q_{O}^{N_2}$ 2nd hr.	$Q_{O}^{N_2}$ 3rd hr.
0	B	− 7·8	− 7·0	− 7·0	3·7	0·5	0	17·9	14·6	—
M/60 oxalate*		− 15·0	− 7·1	− 3·7	6·0	2·6	1·9	5·1	3·9	—
M/50 malonate		− 8·4	− 7·0	− 6·9	1·9	0·9	1·6	12·8	10·0	—
M/50 hydroxymalonate		− 7·8	− 4·9	− 5·4	0·2	1·5	1·1	17·7	14·5	—
0	B	− 7·8	− 7·1	—	1·7	− 1·0	—	19·9	18·4	—
M/50 malonate		− 9·4	− 7·8	—	1·6	− 0·7	—	19·5	18·3	—
0	P	− 8·5	− 8·6	—	—	—	—	—	—	—
M/50 malonate		− 5·2	− 4·1	—	—	—	—	—	—	—
0	B	—	—	—	—	—	—	20·5	18·6	—
M/50 malcate		—	—	—	—	—	—	19·9	18·4	—
M/50 hydroxymalonate		—	—	—	—	—	—	20·0	18·9	—
0	B	—	—	—	—	—	—	19·1	16·2	15·7
M/50 maleate		—	—	—	—	—	—	19·2	15·7	15·0

* Ca^{++}-content of Ringer solution allowed for.

Tale XVII. *Metabolism of various glycolysing tissues in presence of M/50 maleic acid*

		With *M*/50 maleate								Control							
		Q_{O_2}			$Q_{O}^{O_2}$			$Q_{O}^{N_2}$		Q_{O_2}			$Q_{O}^{O_2}$			$Q_{O}^{N_2}$	
Species	Tissue	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.
Guinea-pig	Medulla of kidney (*a*)	− 5·6	− 6·3	− 2·9	10·2	11·5	10·7	24·0	23·0	− 11·8	− 11·0	− 13·7	11·2	9·6	8·9	27·4	26·5
	(*b*)	− 5·4	− 5·5	− 7·3	9·2	10·2	11·5	—	—	− 10·8	− 9·7	− 8·5	12·2	11·1	9·7	—	—
Guinea-pig	Heart	− 4·1	− 1·8	—	4·5	5·2	—	4·4	3·2	− 5·3	− 2·9	—	2·4	1·4	—	6·5	4·4
Rat	Intestinal mucosa	− 7·0	—	—	16·2	—	—	—	—	− 13·3	—	—	17·5	—	—	—	—
Rat	Testis	− 7·5	− 4·5	—	2·1	1·5	—	—	—	− 9·2	− 7·1	—	2·0	0·4	—	—	—
Rat	Spleen (*a*)	− 6·9	− 7·1	—	5·4	8·8	—	—	—	− 14·3	− 13·7	—	3·3	4·8	—	—	—
	(*b*)	− 7·7	− 4·6	—	6·0	9·6	—	7·2	7·0	− 12·9	− 12·8	—	4·2	4·2	—	7·8	7·3
Rat	Jensen sarcoma (*a*)	− 3·5	− 0	—	32·1	27·7	—	30·4	21·9	− 14·4	− 12·4	—	19·9	19·4	—	29·0	21·6
	(*b*)	− 9·0	− 3·5	—	23·9	22·7	—	35·2	30·9	− 7·1	− 4·8	—	23·8	22·9	—	37·0	32·0
Rat	Embryo*	− 8·9	− 5·3	—	9·4	12·7	—	16·5	15·2	− 11·1	− 10·4	—	5·0	4·6	—	19·5	18·6

* Average dry wt. 5 mg.

Influence of metabolites on the maleic acid effect

Certain substrates seem to increase the toxicity of maleic acid. With $M/50$ $l(+)$glutamic acid there is an almost immediate rise of the aerobic glycolysis to the normal anaerobic level followed by a rapid fall. The respiration falls sharply from the beginning (Fig. 5*a*). $d(-)$Glutamic acid has the same effect. With $M/50$ citrate there is a similar fall of respiration. The aerobic glycolysis reaches its peak after 90 min. and declines later (Fig. 5*b*). The presence of $M/50$ pyruvate on the other hand not only keeps the respiration intact, but also abolishes the effect on aerobic glycolysis to a large extent (Fig. 5*c*).

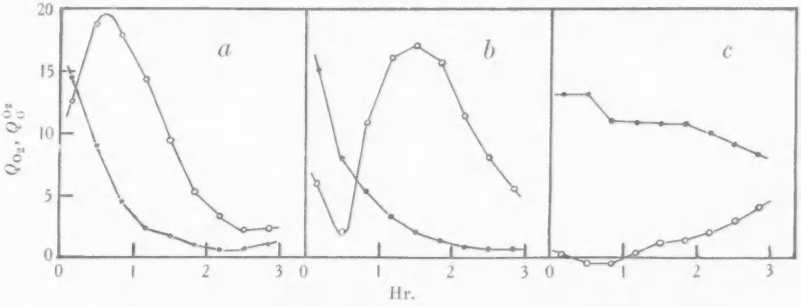

Fig. 5. Respiration and aerobic glycolysis of guinea-pig brain slices. (*a*) with $M/50$ maleate + $M/50$ $l(+)$glutamate; (*b*) with $M/50$ maleate + $M/50$ citrate; (*c*) with $M/50$ maleate + $M/50$ pyruvate. Retention allowed for in all experiments. •——• respiration. o——o aerobic glycolysis.

In Fig. 6 some of the older experiments are reproduced. Since no allowance for retention was then made, only the curves of the aerobic glycolysis which is only slightly affected by the retention are given. $M/50$ ketoglutarate has an effect similar to that of citrate. With $M/50$ fumarate the aerobic glycolysis is diminished.

Although the aerobic glycolysis is hardly increased by $M/100$ maleate addition of $M/100$ glutamate raises it strongly, especially during the 2nd hr. $M/100$ reduced glutathione has a very similar effect (see also Table XV). This is particularly interesting in view of the reaction of maleic acid with thiol compounds discovered by Morgan & Friedmann [1938, 1]. If the effects of maleic acid were due to a destruction of the glutathione of the tissue, the addition of an equivalent amount of glutathione together with the maleic acid should neutralize the effect. What happens is actually the reverse: the effect of maleic acid is accentuated, as is the case with glutamic acid.

Another fact may here be commented upon: the anaerobic glycolysis of brain slices is inhibited by a combination of $M/100$ glutamic acid + $M/100$ maleic acid to approximately the same extent as by $M/100$ glutamic acid alone. Yet under aerobic conditions a strong glycolysis occurs which may be double the glycolysis occurring in the same medium under anaerobic conditions. As pointed out before, the P.M.Q. is here meaningless.

Reversibility: the effect of maleic acid can to some extent be reversed by washing (Fig. 7). After 2 hr. incubation with $M/50$ maleate the slices were removed from the vessels and rinsed several times with Ringer solution; the manometric measurements were then resumed without maleate. In another pair of vessels the observation of the metabolism in presence of $M/50$ maleate was

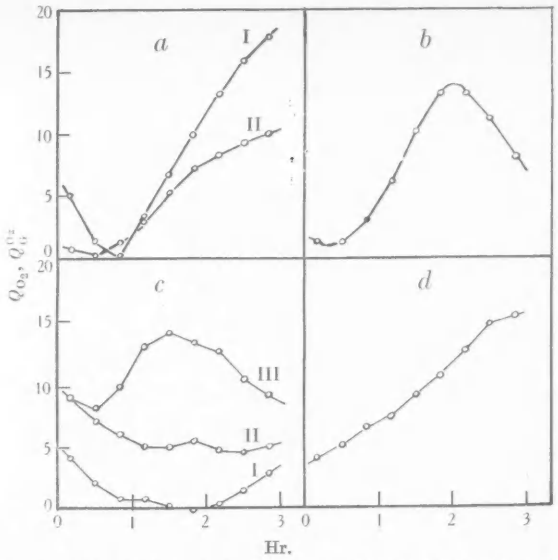

Fig. 6. Aerobic glycolysis of guinea-pig brain slices. (*a*) I, with *M*/50 maleate; II, with *M*/50 maleate + *M*/50 fumarate. (*b*) Same exp. *M*/50 maleate + *M*/50 α-ketoglutarate. (*c*) I, with *M*/100 maleate; II, with *M*/100 *l*(+)glutamate; III, with *M*/100 maleate + *M*/100 *l*(+)glutamate. (*d*) Same exp. *M*/100 maleate + *M*/100 GSH.

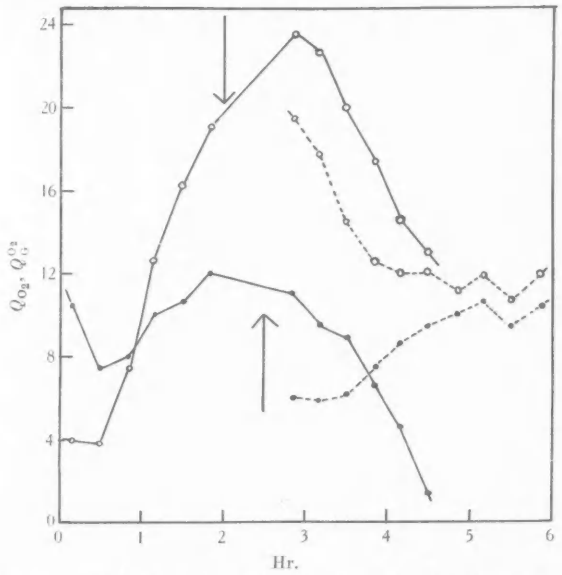

Fig. 7. Reversibility of the maleic acid effect by washing. Guinea-pig brain slices. Downward arrow: slices removed from *M*/50 maleate sol. Upward arrow: slices put back in thermostat after washing. Continuous line: metabolism in presence of *M*/50 maleate. The rise of respiration in the 2nd hr. is probably spurious, since no allowance for retention was made. Broken line: metabolism in bicarbonate-glucose-Ringer after washing. •——• respiration. o——o aerobic glycolysis.

continued until its final destruction. There was, after the washing, some fall of aerobic glycolysis, but not to the normal level, while the respiration recovered almost completely. Another experiment gave a similar result.

(b) The effect on other tissues

The effect of maleic acid is not restricted to brain, but was also observed in other tissues, though to a less marked degree. The inhibition of respiration is usually higher in these tissues (Table XVII). A rise of aerobic glycolysis occurred for instance in embryo, heart and spleen, also in one experiment with Jensen sarcoma, where the respiration was strongly inhibited. Retention was not allowed for in these experiments, but since the aerobic glycolysis was in most cases small or moderate, the errors of respiration values will be small.

(c) The mechanism of the maleic acid effect

(1) The slow development of the maleic acid effect might have been due to poisoning of the "ammonia-binding mechanism" of brain [Weil-Malherbe, 1936] which would lead to an increase of the concentration of NH_4^+ in the tissue. This would also have explained why the effect was so much accelerated by the presence of glutamic acid which would have acted as NH_3 donator. Estimations of NH_3 however showed that no increase occurred after 3 hr. incubation of brain slices with $M/50$ maleate or with $M/50$ maleate + $M/50$ glutamate.

(2) It is of course tempting to seek a connexion between the biological action of maleic acid and the recently discovered reaction with thiol groups [Morgan & Friedmann, 1938, 1, 2]. Indeed Lehmann & Needham [1938] quote the results of Morgan & Friedmann as supporting the theory that the glycolysis of brain needs glutathione as coenzyme. Yet, as has been shown, glutathione does not reverse the action of maleic acid, but, on the contrary, enhances it.

Another compound which is known to react with thiol compounds is iodoacetic acid [Dickens, 1933]. But whereas maleic acid acted as a stimulant of glycolysis in the experiments described, iodoacetic acid is a very potent inhibitor of glycolysis. This action of iodoacetic acid too could not be correlated with the destruction of glutathione [Schroeder *et al.* 1933; cf. also Smythe, 1936]. Needham & Lehmann [1937] could not reverse the inhibition of glycolysis of embryonic tissue caused by iodoacetic acid by the addition of glutathione.

IV. Relevance of the observed phenomena to the theories of the Pasteur reaction

Even if the action of a substance on isolated enzyme systems has been carefully studied, it may be dangerous to apply this knowledge directly to observations made on the intact cell, where its action may be quite different. As for maleic acid little is known about its action on isolated enzymes and nothing that could explain the observed effects. But that they are due to reactions with enzymes seems to be a justifiable conclusion in view of the inactivity of other substances with similar physical properties and a similar or even greater toxicity. This strengthens the case for an enzymic rather than a physico-chemical control of the Pasteur reaction.

Since *l*-glyceraldehyde is in all probability not an intermediate of animal metabolism [Needham & Lehmann, 1937], *l*(+)glutamic, *l*(+)lactic and succinic acids are the only naturally occurring substances which have been shown to inhibit anaerobic glycolysis. Their close connexion with the various

cycles of carbohydrate oxidation may suggest that the degree of saturation of certain dehydrogenases which are parts of these is a factor in the control of the Pasteur mechanism.

Summary

1. NH_4^+ affects the metabolism of brain slices in a way similar to K^+, Rb^+ and Cs^+. $M/30$ NH_4Cl causes increase of respiration, increase of aerobic glycolysis to the anaerobic level and inhibition of anaerobic glycolysis. Some increase of aerobic glycolysis is still seen with concentrations of $M/1000$–$M/3000$ NH_4Cl. The only other tissue where a similar effect of $M/30$ NH_4Cl was observed was spleen. In intestinal mucosa the effect was observed once, but the observation could not be repeated.

2. $M/100$–$M/1000$ $l(+)$glutamic acid inhibits the anaerobic glycolysis of brain slices by 30–70%. $d(-)$Glutamic acid, l-glutamine and dl-β-hydroxyglutamic acid act similarly. The effect is only observed in brain.

3. The effect of glutamic acid is increased by the addition of $M/100$ lactate or $M/100$ succinate.

4. The effect of glutamic acid is largely reversed by $M/100$–$M/1000$ pyruvate.

5. The substances of the glutamic acid group which inhibit the anaerobic glycolysis of brain increase the aerobic glycolysis to values of $Q_{O_2}^{N_2} = 5$–8. This effect, too, only occurs in brain.

6. $M/50$ maleic acid inhibits the respiration of brain slices in bicarbonate-glucose-Ringer to a degree varying in different experiments from 10 to 50%. Aerobic glycolysis rises slowly and reaches the anaerobic level during the 3rd hr. of the experiment. No other simple organic acid has a similar effect.

7. $M/50$ glutamate and $M/50$ citrate accelerate the effect of maleate; $M/50$ pyruvate abolishes it to a large extent.

8. Reduced glutathione does not reverse the effect of maleic acid, but like glutamic acid enhances it.

9. The effect of maleic acid can be partly reversed by washing.

10. In embryo, heart and spleen maleic acid causes a rise of aerobic glycolysis, which in some cases reaches the level of the anaerobic glycolysis.

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CCXC. THE FORMATION OF GLUCOSE FROM ACETOACETIC ACID IN RAT KIDNEY

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In an earlier paper [Weil-Malherbe, 1938] the formation of a reducing substance from acetoacetic acid by rat kidney slices was reported. It will be shown that this substance is very probably glucose.

This observation is of interest in view of the still unsettled question of the transformation of fat into carbohydrate. Blixenkrone-Møller [1938] found an increase of liver glycogen after perfusion of cat liver with Na butyrate, but not with β-hydroxybutyrate. Haarmann & Schroeder [1938] described the formation of reducing substances from β-hydroxybutyric and dihydroxybutyric acids, but no evidence was brought forward by which these substances could be conclusively identified as carbohydrates. How important this identification is will be obvious in the light of the fact that, while the reducing compound formed by rat kidney slices from the first substance is glucose, it is mainly α-hydroxyacetoacetic acid or acetol in the second case.

Benoy & Elliott [1937] showed that a considerable increase of total carbohydrate is found after the aerobic incubation of rat kidney slices with pyruvate and, to a smaller extent, also with lactate, succinate or malate, though no synthesis of glycogen occurs in kidney.

Experimental

Estimation of reducing power. The reagent of Somogyi [1937] was used throughout. An empirical calibration curve for glucose was determined, which was almost linear except for very low concentrations, 1 ml. $M/100$ I_2 indicating 30 μl. glucose. In the case of acetol 1 ml. indicates 112 μl.

If not stated otherwise, proteins and interfering substances were removed by $Cd(OH)_2$ precipitation [Miller & Van Slyke, 1936]. The reagent used contained 13·0 g. $3CdSO_4$, $8H_2O$ and 63·5 ml. N H_2SO_4 in 100 ml. 0·8 ml. of this solution + 1·1 ml. N NaOH were added per 10 ml.

The results are expressed in the conventional Q-units

$$\left(\text{e.g. } Q_{\text{glucose}} = \frac{\mu\text{l. glucose}}{\text{mg. dry wt.} \times \text{hr.}}\right).$$

I. *Formation of a reducing substance*

After aerobic incubation of rat kidney slices with acetoacetic acid an increased amount of a reducing substance is found in the suspension fluid. The magnitude of the effect may be seen from Table I where the results are calculated on the assumption that the reducing substance is glucose.

Table I. *Formation of reducing substance by slices of rat kidney cortex*

Bicarbonate-saline. $O_2 + 5\%$ CO_2. 3 hr. incubation

Exp.	Substrate	$Q_{glucose}$
1	0	0·11
	M/50 acetoacetate	0·41
2	0	0·12
	M/50 acetoacetate	0·34
3	0	0·07
	M/50 acetoacetate	0·26

The amount of reducing substance slowly increases with time (Fig. 1).

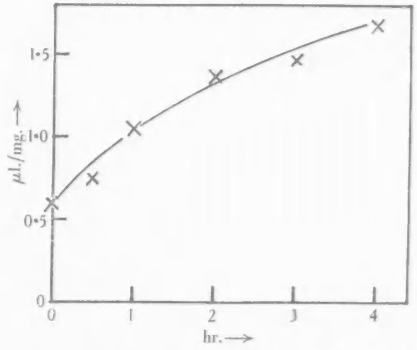

Fig. 1. Increase of reducing substance (calc. as glucose) with time.

No soluble reducing substances were found after aerobic incubation of acetoacetic acid with slices of brain, heart, Jensen rat sarcoma or with testicular tissue.

II. *Identification of the reducing substance as glucose*

(1) The reducing substance was found not to be volatile from neutral, acid or alkaline solution. The skatole test of Jordan & Pryde [1938] gave a vivid purple colour after heating to 80°, while it was negative at 40°, thus indicating the presence of an aldohexose.

Each of two vessels contained approx. 200 mg. dry wt. of slices suspended in 25 ml. bicarbonate-saline. The gas space was filled with 5 % CO_2 in O_2. One of the vessels also contained *M*/50 aceto-acetate as substrate. After 3 hr. incubation the tissue was removed and 3 ml. $CdSO_4$ and 4 ml. *N* NaOH were added. The filtrate was neutralized and evaporated *in vacuo* to dryness. The residue was dissolved in 1 ml. water. 10 ml. conc. HCl and 10 mg. recryst. skatole were then added and the solutions put in a water bath at 37·5° for 30 min. No colour developed. After transfer to a water bath at 80° a deep violet colour soon developed in the solution with acetoacetic acid. The colour in the control appeared much later and was very much feebler. A pure solution of acetoacetate showed no colour at all.

(2) One of the most characteristic properties of glucose is its fermentability by yeast. Instead of estimating the reduction before and after fermentation, as is the usual procedure, the CO_2 of fermentation was measured manometrically thus preventing the possible loss of reducing substances by processes other than fermentation. The ratio of μl. glucose by reduction : μl. CO_2 of fermentation was

simultaneously determined in the experimental solutions and in a standard solution of glucose. The latter was made up with an amount of saline equal to that used for the experiment and was subsequently subjected to an identical treatment so that the final salt concentration in the standard and experimental solutions was the same. In addition a control solution without glucose containing an equal salt concentration was prepared. This was found necessary, as the blank gas evolution was somewhat increased in the high salt concentration. Also the yield of CO_2 of fermentation which was 90–92 % in $M/30$ KH_2PO_4 (reduction/fermentation = 0·55) was slightly lower and varied from 82 to 90 % (reduction/fermentation = 0·61–0·56) according to the method of deproteinization and the salt concentration which it entailed. The yield was highest after a simple kieselguhr filtration though in the experimental solutions the reduction value greatly exceeded the value calculated from the fermentation, especially in absence of the substrate (Table II). The lowest yield (82 %) was obtained after a combined tungstic acid-$Cd(OH)_2$ deproteinization, but by this procedure most of the non-sugar reducing material appeared to be eliminated and the ratio of reduction/fermentation was almost identical in the experimental and standard solutions. This is strong evidence for the identity of the substance estimated in both cases.

Table II. *Formation of fermentable reducing substance by slices of rat kidney cortex*

Exp.	Substrate	Method of deproteinization	Q_{glucose} calc. from CO_2 of fermentation	Reduction/fermentation	Reduction/fermentation in standard of pure glucose
1	0	Kieselguhr	0·04	2·18	0·56
	$M/50$ acetoacetate		0·23	0·78	
2	0	$Cd(OH)_2$	0·08	0·93	0·59
	$M/50$ acetoacetate		0·29	0·68	
3	0	Na_2WO_4 + $Cd(OH)_2$	0·07	0·62	0·61
	$M/50$ acetoacetate		0·28	0·61	

Each of two vessels contained 130–160 mg. dry wt. of slices suspended in 25 ml. bicarbonate-saline in an atmosphere of $O_2 + 5\%$ CO_2. One of the vessels (vessel II) contained $M/50$ acetoacetate. Duration of the experiment: 3 hr.

Deproteinization. Exp. 1: 1 ml. 10 % H_3PO_4 (10 vol. syrupy H_3PO_4 in 100 vol. solution) was added to vessel I and 1·5 ml. 10 % H_3PO_4 to vessel II. After ½ hr. in a boiling water bath (see below) the solutions were brought to *p*H 5–6 by the addition of 1 ml. and 1·5 ml. N NaOH respectively, and filtered after addition of kieselguhr.

Exp. 2: 3 ml. $CdSO_4$ sol. and 4 ml. N NaOH added and centrifuged.

Exp. 3: 2·5 ml. N H_2SO_4 and 2·5 ml. 10 % Na_2WO_4 were added and the sol. centrifuged after 10 min. 2 ml. $CdSO_4$ sol. and 4 ml. N NaOH were then added to the supernatant solution and it was again centrifuged.

The deproteinized solutions were placed in a boiling water bath for 30 min. to decompose excess acetoacetic acid. After cooling they were adjusted to *p*H 5–6 by the addition of 10 % H_3PO_4 and evaporated to dryness *in vacuo*. The dry residue was taken up in 5 ml. water. 2 ml. were transferred to the manometer vessel for the measurement of fermentation, while the remainder was used for estimation of reducing power. The side bulb of the manometer vessel contained 0·5 ml. of a 10 % suspension of well washed D.C.L. baker's yeast in $M/30$ KH_2PO_4. The gas space was filled with 5 % CO_2 in N_2, freed from O_2 over hot copper. Control vessels containing salt solutions with and without a known amount of glucose were set up simultaneously. The measurement was carried out at 25°.

(3) Finally glucose was estimated by the method of Butkevič & Gajevskaja [1935] based on the colorimetric estimation of the osazone formed from glucose. The conditions worked out by these authors were rigorously adhered to, except that a glucose standard solution was used for the preparation of the colorimetric standard (Table III).

Table III. *Formation of osazone-yielding substance by rat kidney slices*

Substrate	$Q_{glucose}$ estimated by reduction	$Q_{glucose}$ estimated by osazone method
0	0·12	0·04
$M/50$ acetoacetate	0·30	0·21

The arrangement of the experiment was as described under (1). After $Cd(OH)_2$ precipitation acetoacetic acid was decomposed by boiling and acetone removed by evaporation *in vacuo* as under (2). The residue was taken up in 10 ml. water, of which 3 ml. were used for estimation of reducing power and the rest for osazone formation. No hydrazone was formed with a pure solution of acetoacetic acid treated in this way.

The experiments described show that, on incubation of rat kidney slices with acetoacetic acid, a soluble, reducing, fermentable and osazone-yielding substance is formed in amounts which are from 3 to 7 times larger than in the control. The relative increase is larger with the more specific fermentation and osazone methods than with the unspecific reduction method. Since the skatole test which at 80° is specific for aldohexoses was strongly positive, it can be safely assumed that the substance in question is indeed glucose.

III. *Increase of total fermentable carbohydrate*

It remained to decide whether the effect observed was due to a sparing action of acetoacetic acid on preformed carbohydrate or to a true synthesis of glucose. Analyses of the total fermentable carbohydrate showed a considerable increase after incubation with acetoacetic acid, thus indicating a synthesis (Table IV).

Table IV. *Increase of total fermentable carbohydrate in rat kidney slices*

	μl. total fermentable carbohydrate (as glucose) per 100 mg. wet wt.			
	With $M/50$ acetoacetate		Without substrate	
Exp.	Initial	Final	Initial	Final
1	9·2	14·5	8·5	8·4
2	8·1	21·8	9·2	8·0
3	10·0	23·3	8·0	6·5
4	8·8	14·5	7·4	15·4
5	8·8	17·6	6·6	12·2

Slices from 6 rat kidneys were distributed into 4 tared, stoppered weighing bottles containing saline and weighed. They were then transferred to vessels 1–4 (about 500 mg. slices (wet wt.) per vessel).

Vessel ...	1	2	3	4
Bicarb. saline (ml.)	8	8	8	8
0·4 M acetoacetate (ml.)	0	0	0·4	0·4
Addition of 0·5 ml. 10 N H_2SO_4	t_0	3 hr.	t_0	3 hr.

Gas space: 5 % CO_2 in O_2.

After the experiment the vessels were put in a boiling water bath for 10 min. The contents were then transferred to a mortar, thoroughly ground and finally poured into a large test tube.

Vessel and mortar were rinsed 3 times with 1 ml. N H_2SO_4. The test tube was heated in a boiling water bath for 3 hr. After cooling, 2·5 ml. 10% NaOH and 1·3 ml. 10% sodium tungstate were added, the volume measured and the solution centrifuged. 1 ml. $CdSO_4$ sol. and 0·4 ml. 20% NaOH were added to the measured supernatant fluid and it was again centrifuged. The clear solution was made up to 10 ml. or, if more than 10 ml., its volume noted. The estimation of reducing power was carried out with 5 ml. before, and with 5 ml. after fermentation. For fermentation 5 ml. of a 10% suspension of well washed baker's yeast were centrifuged and the solution to be fermented was added to the residue. After thorough mixing the tube was left at room temp. for 15 min. before it was again centrifuged and the reduction determined in the supernatant solution.

IV. *The nature of the reducing substance formed from dihydroxybutyric acid by rat kidney slices*

Previously [Weil-Malherbe, 1938] experiments with rat kidney slices were described showing the disappearance of dihydroxybutyric acid and the simultaneous formation of a reducing compound. The latter was assumed to be largely acetol, since the intensity of the fluorescence test with *o*-aminobenzaldehyde roughly corresponded to that obtained with a solution containing an amount of acetol equivalent to the reduction value of the experimental solution. It was however desirable, in view of the results obtained with acetoacetic acid, to adduce further evidence and to decide whether glucose was formed besides acetol and if so in what quantity. The reducing power was therefore determined in the experimental solution before and after evaporation to dryness *in vacuo*, and the soluble fermentable carbohydrate in the non-volatile residue. The same analytical procedure was carried out with a control (no substrate) and with an experiment with acetoacetic acid as substrate. The decrease of reducing power after evaporation was calculated as acetol, that after fermentation as glucose.

Table V. *Formation of acetol and glucose by rat kidney slices*

Exp.	Substrate	Q_{acetol}	$Q_{glucose}$
1	0	0	0·01
	$M/50$ *dl*-threo-1:2-dihydroxybutyrate	2·03	0·12
	$M/50$ acetoacetate	0	0·23
2	0	0	0·14
	$M/50$ *dl*-threo-1:2-dihydroxybutyrate	2·3	0·11
	$M/50$ acetoacetate	0·07 (?)	0·31

Each of three vessels contained about 80 mg. slices (dry wt.) suspended in 8 ml. bicarbonate-saline. There was no substrate in the first, $M/50$ *dl*-threo-1:2-dihydroxybutyrate in the second and $M/50$ acetoacetate in the third vessel. The gas space was filled with 5% CO_2 in O_2. After an incubation of $2\frac{1}{2}$ hr. the solution was acidified with 0·3 ml. 2 N HCl, the tissue was removed and the solution neutralized to litmus with about 0·33 ml. N NaOH. A little kieselguhr was added and the solution was heated in a boiling water bath for 5 min. The clear filtrate was made up to 10 ml.: 3 ml. were taken for the estimation of reducing power immediately and the remaining 7 ml. were evaporated *in vacuo* to dryness. 2 ml. water were added to the dry residue, again evaporated and this process was repeated once more. The residue was taken up in 10 ml. water: 5 ml. were used for reduction before, and 5 ml. after fermentation, as described. A large proportion of the volatile reducing substance was recovered in the distillate from the solution containing dihydroxybutyric acid, whereas the distillates from the other two solutions did not contain any reducing substance.

It is quite clear from Table V that the results fully confirm the earlier observations and conclusions. A considerable quantity of a volatile reducing substance is formed in the experiment with dihydroxybutyric acid, but none in the control or in the acetoacetate experiment. The small amount of "acetol" found in Exp. 2 in the presence of acetoacetic acid is within the experimental error and is therefore insignificant. On the other hand much more soluble

fermentable carbohydrate is found in the presence of acetoacetic acid than in the other experiments. As far as the formation of glucose from dihydroxybutyric acid is concerned, there was no increase over the control in Exp. 2. Almost the same value as in Exp. 2 was observed in Exp. 1, but here the amount found in the control was unusually low and the apparent increase may well have been due to a sparing action.

There is therefore hardly any doubt that the metabolism of dihydroxybutyric acid and that of acetoacetic acid follow different routes and our earlier conclusion that neither dihydroxybutyric nor hydroxyacetoacetic acid is an intermediary in the oxidation of acetoacetic acid is confirmed.

V. *The mechanism of the formation of glucose from acetoacetic acid*

Benoy & Elliott [1937] find that the greatest synthesis of carbohydrate in rat kidney is observed in presence of pyruvic acid, and they conclude that pyruvic acid is an intermediary in the process of carbohydrate synthesis from other substrates, such as lactic, succinic and malic acids and alanine, all of which are known to be convertible into pyruvic acid.

The quantities of soluble reducing matter (calculated as glucose) found in the suspension fluid after 2 hr. incubation of rat kidney slices in presence and in absence of *l*(+)-alanine, acetoacetic acid and pyruvic acid are presented in Fig. 2, showing the enormous superiority of pyruvic acid. Though the conversion of acetoacetic acid into pyruvic acid has not yet been established, such a reaction may well occur. It is however extremely improbable that α-hydroxyacetoacetic acid is an intermediary in the process.

Should the oxidative conversion of acetoacetic into pyruvic acid be confirmed, it would mean another example of a cyclic oxidation of pyruvic acid, since pyruvic acid is converted into acetoacetic acid in many tissues (cf. Embden & Oppenheimer [1912], Annau [1934] and Edson [1935] for liver; Annau [1935] for liver, muscle and kidney; Weil-Malherbe [1936] for brain; Krebs & Johnson [1937] for muscle, heart, liver and kidney).

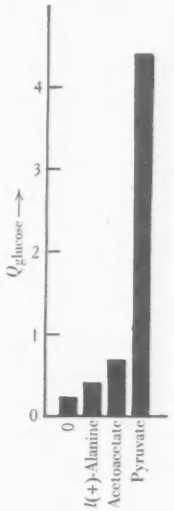

Fig. 2. Formation of reducing substance (cal. as glucose) from various substrates.

SUMMARY

If slices of rat kidney cortex are incubated with acetoacetic acid aerobically, an increased quantity of a reducing substance appears in the suspension fluid. This substance has been identified as glucose by the colour reaction with skatole, by its fermentability by yeast and by the formation of an osazone. An increase of the total fermentable carbohydrate indicates a true synthesis of carbohydrate from acetoacetic acid.

It is confirmed that no acetol is formed from acetoacetic acid, whereas the reducing substance formed from dihydroxybutyric acid is largely acetol, only a minute fraction being glucose.

It is suggested that pyruvic acid is an intermediary in the synthesis of glucose from acetoacetic acid.

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CCXCI. THE BIOCHEMISTRY OF SILICIC ACID

VII. DEPRESSION OF THE APPARENT SOLUBILITY OF SILICA IN BODY FLUIDS

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(*Received 29 October 1938*)

The depression of solubility of SiO_2 through the agency of various metals and metallic oxides, notably Fe and Al, was briefly referred to in a former communication [King & McGeorge, 1938]. It was suggested that the apparent decrease in solubility of quartz and amorphous SiO_2 was due to precipitation of the dissolved silicic acid as the insoluble silicate of the metal. The phenomenon has now been explored further, and the effects of various mineral dusts on the solution of SiO_2 from quartz have been investigated.

Most of the mineral silicates are much less soluble in blood serum and ascitic fluid than are the free forms of SiO_2. Kaolin is particularly insoluble. In a mixture of kaolin and quartz powder suspended in a liquid it might be expected that the amount of SiO_2 dissolved would be conditioned by the most active member of the mixture, and that in the presence of an excess of the mixed powder the solubility would approach that of quartz powder taken alone. Naturally occurring kaolins, such as those represented in shales, contain variable, sometimes large, amounts of free SiO_2, and yet these natural stone dusts have an exceedingly low solubility. The amount of free SiO_2 represented in a sample of stone dust would lead to much more solution of silica if suspended alone in the same volume of liquid. This fact was brought out in the previous communication where it was shown that a stone dust (shale—used for mine dusting) had a solubility of less than 1 mg./100 ml. although it contained about 40 % free SiO_2, whereas the 0·8 g. free SiO_2 present in the 2 g. stone dust taken for 100 ml. ascitic fluid would if taken separately dissolve to the extent of 9 mg./100 ml. The figures set out in Table I demonstrate these points more fully. The methods used in this investigation—preparation and examination of dusts, solution experiments, separation of solid from liquid phase, determination of dissolved SiO_2 etc.—were those described in the previous communication [King & McGeorge, 1938]. The silicate minerals are much less soluble than quartz in the ascitic fluid used,[1] and the stone dusts are only slightly soluble despite their free SiO_2 contents which in some cases are large. The SiO_2 dissolved from the coal and coal-mine dusts is, of course, related to the silicate minerals contained in them, and is of the same order as that from the stone dusts.

The apparent depression of the solution of free SiO_2 contained in the stone dusts suggested that the effect might be extended to quartz admixed with the stone dust. This proved to be the case as is shown in Table II. The addition of increasing amounts of stone dust to 2 g. quartz suspended in 100 ml. ascitic fluid led to a progressive decrease in the SiO_2 dissolved, from 7 mg. SiO_2/100 ml. to

[1] This ascitic fluid was of a more alkaline reaction (pH 8·0) than that used in the previous investigation, and the figures for solubility of the various dusts are hence not identical with those recorded in the previous paper.

Table I. *Dissolution of silica from mineral dusts*

2 g. dust suspended in 100 ml. ascitic fluid at 37°

	Mg. SiO_2 per 100 ml.		
Mineral dusts:	1 day	3 days	6 days
Quartz	4·4	5·4	7·0
Sericite	—	1·7	1·6
Chlorite (Mass.)	—	1·1	1·4
Chlorite (Saxony)	—	1·0	1·2
Kaolin	0·1	0·1	0·1
Biotite	—	1·0	1·1
Stone dusts:			
D. and C., S. 194* (39% free SiO_2, 10·8% Al)	0·5	0·6	0·6
C. and W., S. 20* (7% free SiO_2, 13·5% Al)	0·6	0·9	1·5
P. and P., S. 31* (21% free SiO_2, 9·1% Al)	1·4	1·5	1·3
Somerset Greys† (49% free SiO_2, 7·2% Al)	2·2	2·1	2·0
Pennant Rock† (42% free SiO_2, 5·9% Al)	2·4	2·5	2·2
Air-borne coal-mine dusts:			
Am. D. (16% ash)	1·2	1·4	1·4
Am. E. (39% ash)	1·5	1·4	1·1
Am. I. (18% ash)	1·2	1·4	1·8
Am. J. (25% ash)	0·8	1·3	1·4
Coal dusts:			
Am. anthracite (3·9% ash)	0·9	1·5	2·0
Cy. bottom coal (2·5% ash)	1·5	2·1	2·1
Ba. soft coal (4·5% ash)	1·7	1·6	1·8
Yn. anthracite (6·3% ash)	1·5	1·2	1·4

* Shale dusts used for dusting underground.
† Coal-measure sandstones, produced by drilling.

Table II. *Dissolution of silica from mixtures of quartz and mineral dusts*

mg. SiO_2 dissolved by 100 ml. ascitic fluid

		1 day	3 days	6 days
2%*	Quartz (alone)	4·4	5·4	7·0
2%	D. and C. stone dust (alone)	0·5	0·6	0·6
2%	Quartz + 1% stone dust	3·2	3·4	4·6
2%	Quartz + 2% stone dust	1·8	1·7	2·4
2%	Quartz + 4% stone dust	1·6	1·4	1·7
2%	Yn. coal dust (alone)	1·5	1·2	1·4
2%	Quartz + 2% coal dust	2·9	2·6	3·7
2%	Quartz + 4% coal dust	2·2	1·8	2·2
2%	Kaolin (alone)	0·1	0·1	0·1
2%	Quartz + 2% kaolin	2·7	4·6	4·5
2%	Quartz + 4% kaolin	1·8	2·3	2·4
2%	Quartz + 1% Al_2O_3	0·4	0·3	0·3

* The % figures (e.g. 2% quartz) denote the number of g. dust per 100 ml. ascitic fluid.

1·7 mg. when twice as much stone dust as quartz was present. The phenomenon, moreover, seemed to be a general one, as a similar depression was obtained with several stone dusts and silicate minerals (Table III).

Coal dust, and the air-borne dusts from coal mines, appear to depress SiO_2 solubility in proportion to their silicate mineral content. Pure carbon (Kahlbaum's "carbo activ") and charcoal of low ash content have no effect on the solution of quartz. The depressions seen in Table IV would seem, therefore, to depend on the presence of stone dust in the coal. One of the coal dusts (Cy.) differs from the others in that it causes an increase in the amount of SiO_2 dissolved. This dust probably differs also from the others in the nature of its siliceous constituents.

Table III. *Effect of mineral dusts on the dissolution of quartz*

% reduction in amount of SiO_2 dissolved from 2 g. quartz in 100 ml. of ascitic fluid

	1 day	3 days	6 days
Sericite (2 g.)	—	49	61
Biotite (2 g.)	—	79	81
Chlorite (Mass.) (2 g.)	—	80	78
Chlorite (Saxony) (2 g.)	—	81	81
Kaolin (1 g.)	3	0	18
Kaolin (4 g.)	59	62	64
Al_2O_3 (1 g.)	90	96	96
Al_2O_3 (4 g.)	96	98	97
D. and C. stone dust (1 g.)	28	38	27
D. and C. stone dust (2 g.)	58	69	66
C. and W. stone dust (1 g.)	28	20	32
C. and W. stone dust (2 g.)	39	38	40
P. and P. stone dust (1 g.)	44	37	50
P. and P. stone dust (2 g.)	55	61	64
Somerset Greys (1 g.)	23	36	37
Somerset Greys (2 g.)	28	44	57
Pennant Rock (1 g.)	37	44	56
Pennant Rock (2 g.)	42	44	65

Table IV. *Effect of coal dust on the dissolution of quartz*

2 g. quartz, 4 g. coal dust per 100 ml. ascitic fluid; % reduction in amount of SiO_2 dissolved

	1 day %	3 days %	6 days %
Am. D. air-borne dust (16% ash)	23	6	21
Am. E. air-borne dust (39% ash)	53	63	75
Am. I. air-borne dust (18% ash)	33	50	54
Am. J. air-borne dust (25% ash)	71	64	71
Am. anthracite (3·9% ash)	40	30	35
Ba. soft coal (4·5% ash)	54	55	62
Yn. anthracite (6·3% ash)	51	69	69
Cy. bottom coal (2·5% ash)	(11)	(34)	(5% increase)

Depression of solubility of SiO_2 by these dusts may be dependent on adsorption of the dissolving silicic acid, on its precipitation as an insoluble silicate, or on the coating of the silica particles by some constituent of the depressor. In Table V are shown the results of adding silicate and mixed stone dusts to a saturated solution of SiO_2 in ascitic fluid which had been previously filtered free of all suspended particles. The depression of the amount of dissolved SiO_2 by the various mineral dusts is here clearly a case of precipitation or of adsorption. The SiO_2 dissolved in the liquid may react with some constituent of the mineral dust to form an insoluble precipitate. The coating of the solid particles of SiO_2 by a layer of the "depressor", e.g. of $Al(OH)_3$, would account for the depression of solubility shown in Table III, but this sort of mechanism cannot furnish a satisfactory explanation of the precipitation of dissolved SiO_2 shown in Table V. Further work on the composition of the solid phase of these mixtures should reveal an enrichment of the dust in SiO_2, and may possibly reveal the nature of the change taking place. Most mineral silicates are not of rigidly uniform composition, and it is possible that many of them may be capable of reacting with excess free SiO_2 with a consequent formation of a complex of higher SiO_2 content than the original.

The precipitation of SiO_2 from its solution by dusts from coal mines is shown in Table V. The results are somewhat irregular. No well-defined relationship is seen between the amount of depression of the SiO_2 in solution and the ash content. But the precipitation of dissolved SiO_2 has nevertheless almost certainly been

Table V. *Precipitation of dissolved silica by mineral dusts*

Decrease of dissolved SiO_2 in a "saturated solution" of quartz in ascitic fluid, 9 mg./100 ml., due to added dust

	g. dust added per 100 ml.	% decrease in dissolved SiO_2		
		1 day	3 days	6 days
Sericite	2	—	24	17
Biotite	2	—	33	35
Chlorite (Mass.)	2	—	76	75
Chlorite (Saxony)	2	—	69	66
Kaolin	2	32	29	26
	4	38	26	32
Al_2O_3	2	94	94	94
	4	95	95	96
D. and C. stone dust	2	24	31	38
	4	36	44	54
C. and W. stone dust	2	40	53	—
	4	46	50	—
P. and P. stone dust	2	27	25	18
	4	37	21	27
Somerset Greys	2	11	10	24
	4	22	20	30
Pennant Rock	2	8	5	12
	4	7	14	30
Am. D. air-borne dust	2	6	1	0
	4	9	4	4
Am. E. air-borne dust	2	4	21	20
	4	14	18	21
Am. I. air-borne dust	2	8	12	—
	4	16	23	12
Am. J. air-borne dust	2	20	—	7
	4	27	21	21

brought about by the minerals contaminating the coal. It is possible that adsorption of silicic acid by the carbonaceous particles of coal dust may play a part in the retardation of solution of SiO_2 and in the precipitation of SiO_2 already existing in solution, but this is unlikely since activated charcoal is without effect. The carbonaceous constituents of the coal may, however, affect the solubility of SiO_2 in an indirect manner, through an interaction (e.g. by adsorption) with some of the constituents of the ascitic fluid. Whether an adsorptive effect plays a part in the phenomenon of depression by coal dust of SiO_2 solubility, as well as the possibility of a reaction of the dissolved SiO_2 with different constituents of the dust will form the subject of further investigation.

The stone dusts used in this study[1] were samples of dust which have long been used for the stone-dusting of coal mines in Great Britain with the purpose of diminishing the hazard of explosion. Industrial experience during many years has led to the belief that stone-dusting in coal mines has not introduced or increased the silicosis hazard. Indeed the belief has slowly evolved that stone-

[1] The stone dusts were furnished by Dr A. G. R. Whitehouse, of the Safety in Mines Research Laboratory, who finds them to have a depressing action on the solution of free SiO_2 in Na_2CO_3 solution [1938], and with whom the authors have been fortunate to be in collaboration. The mineral dusts—sericite, chlorite, biotite and kaolin—were kindly given us by Dr A. Brammall, and the air-borne coal-mine dusts by Prof. H. V. A. Briscoe.

dusting actually diminishes the risk of silicosis in mines [cf. Haldane, 1917; 1931], and that certain mineral silicates may off-set the action of quartz in its relation to the disease. Recent experimental work on the effect of mineral dusts on the lungs and other tissues of animals [Gardner, 1938] has appeared to indicate that many mineral silicates may modify and even retard the action of free SiO_2. The finding that certain stone dusts modify the silicosis hazard may have its explanation in the experimental findings reported here, but at the present time it can only be interpreted as an interesting parallel which merits further study.

SUMMARY

The presence of shale and of some other silicate minerals may markedly diminish the amount of SiO_2 dissolved from a mixed rock dust in ascitic fluid. The amount of SiO_2 dissolved from quartz is reduced, and SiO_2 is removed from solution in the presence of these silicates.

The authors are indebted to the Medical Research Council for a grant covering the expenses of this investigation.

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CCXCII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

LIX. SPINULOSIN (3:6-DIHYDROXY-4-METHOXY-2:5-TOLUQUINONE) A METABOLIC PRODUCT OF A STRAIN OF *ASPERGILLUS FUMIGATUS* FRESENIUS

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Anslow & Raistrick [1938, 1] reported the isolation, from cultures of a strain of *Aspergillus fumigatus* Fresenius grown on Raulin-Thom medium at 24°, of fumigatin, which they showed to be 3-hydroxy-4-methoxy-2:5-toluquinone. We have now shown that a different strain of *A. fumigatus*, when grown under the same cultural conditions, produces 6-hydroxyfumigatin, i.e. spinulosin (3:6-dihydroxy-4-methoxy-2:5-toluquinone). Spinulosin was first reported by Birkinshaw & Raistrick [1931] as a metabolic product of *Penicillium spinulosum* Thom and its constitution was settled by synthesis by Anslow & Raistrick [1938, 2].

The formation of spinulosin by *A. fumigatus* and *P. spinulosum* adds one more to the small but growing number of examples of species of moulds in different genera which give rise to the same metabolic product. The formation of fumigatin by one strain and of spinulosin by a different strain of the same species of mould is of interest mycologically since, if, as we believe, fumigatin and spinulosin function as oxidation-reduction systems in the vital processes of the moulds which produce them, then their formation points to quite different "oxidation levels" in two authentic strains of the same species of mould. It is of interest to note in this connexion that Anslow & Raistrick [1938, 1] found that only one of six strains of *A. fumigatus* examined gave fumigatin and none produced spinulosin.

Experimental

Culture

The culture used was, morphologically, undoubtedly a strain of *Aspergillus fumigatus* Fresenius. It was isolated by Mr G. Smith in October 1936 from dried Kentish hops, and bears the L.S.H.T.M. Catalogue number A 49. Thom & Church [1926], in their diagnosis of *A. fumigatus*, give "Colonies on Czapek's solution agar in some strains strictly velvety, in others with varying amounts of tufted aerial mycelium up to felted floccose forms." It is interesting to note that colonies of *A. fumigatus* A 49 are almost strictly velvety, sporing readily, whilst *A. fumigatus* A 46, the strain used by Anslow & Raistrick [1938, 1], for the production of fumigatin, gives colonies which are densely floccose, sporing very tardily. The two strains thus represent almost the extreme morphological range of this species.

Cultural conditions

The culture medium used was a Raulin-Thom solution of the following composition: glucose, 75 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4$, $7H_2O$, 0·07 g.; $FeSO_4$, $7H_2O$, 0·07 g.; distilled water to 1500 ml. This medium was distributed in 350 ml. amounts in each of 100 l1. conical flasks, sterilized, sown with a spore suspension of *A. fumigatus*, A. 49, and incubated in the dark at 24°.

At the end of the incubation period (25–26 days) the flasks were uniform in appearance, having a crumpled grey mycelium with dark green sporing patches and a grey reverse. The metabolism solution, which was dark purple-red in colour, contained only 0·04 % of residual glucose (by polarimeter) and its *p*H was 5·86. The purple colour was discharged on acidification and became yellow. The solution rapidly reduced permanganate and gave a brown colour with ferric chloride. 50 ml. liberated iodine, from acidified KI solution, equivalent to 4·87 ml. *N*/100 $Na_2S_2O_3$.

Isolation and purification of spinulosin

The filtered metabolism solution from 90 flasks was acidified by the addition of conc. HCl (20 ml./l.) and was then extracted twice, in 2 l. lots, with an equal volume of ether, the ether used for the second extraction of one lot of metabolism solution being used again for the first extraction of another lot. The ethereal extracts were washed with a little water, dried over anhydrous $MgSO_4$ and evaporated to about 1 l. This ethereal solution was now extracted 4 times with a total volume of 200 ml. of a buffer solution (*p*H 7·0) made by mixing 50 ml. *M* KH_2PO_4 with 29·6 ml. *N* NaOH and diluting with water to 100 ml. The extracted ethereal solution was redried and evaporated to dryness, giving 1·7 g. of a gummy residue. No evidence of the presence in this fraction of dihydrospinulosin could be obtained, and nothing crystalline could be isolated except 0·15 g. of an unidentified yellow solid, M.P. 185–190°. The combined buffer solution extracts, which were deep purple in colour, were acidified with conc. HCl and extracted with ether; the ethereal extract was dried and evaporated, giving 2·3 g. of a crystalline, slightly sticky, purple-black residue of crude spinulosin. This was purified by crystallization from toluene, followed by sublimation in a high vacuum and finally by recrystallization from toluene. The final product (0·93 g.) was obtained as lustrous purple-black plates and was identified as spinulosin by its M.P. 200°, alone or in admixture with an authentic specimen, by its colour reactions (a deep pure blue with cold conc. H_2SO_4, a bluish-purple with 2*N* NaOH, and a very dark rich brown with $FeCl_3$ in alcohol), and by analysis. (Found (Weiler): C, 52·32, 52·39; H, 4·34, 4·40; O.CH_3, 16·9, 16·85 %. Calc. for $C_8H_8O_5$: C, 52·17; H, 4·38; 1 O.CH_3, 16·9 %.)

SUMMARY

Spinulosin (3:6-dihydroxy-4-methoxy-2:5-toluquinone), previously reported as a metabolic product of *Penicillium spinulosum* Thom, has now been isolated from cultures of a strain of *Aspergillus fumigatus* Fresenius. This finding contrasts with the fact that a different strain of *A. fumigatus* gives fumigatin (3-hydroxy-4-methoxy-2:5-toluquinone).

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CCXCIII. THE DISSOCIATING POWER OF SALTS OF FATTY ACIDS

PRELIMINARY PAPER

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This work had its origin in the observation that the dissociative change in colour induced in lobster shell by alcohol was partly reversible. This was made shortly before the appearance of the preliminary paper of Stern & Salomon [1937], who have drawn attention to the reversible colour change occurring upon mild heating of the chromoprotein, ovoverdin, from lobster eggs, and have explored part of this interesting field [1938]. Denaturation of ovoverdin, as ordinarily seen, involves the two processes (*a*) of dissociation of the astacin ester from the associated protein, giving the colour change green to red, and (*b*) of denaturation (with coagulation) of the protein itself. We deal here only with the dissociation, making the assumption that the colour change represents a change in the state of combination of the carotenoid with the protein. We found that this dissociation can be quickly induced at neutral reaction by traces of the Na salts of oleic and of other fatty acids, and further that these acids, when added to a respiring brain *brei*, inhibit respiration. It is suggested here that the two sets of observations are related, and that this inhibition is caused by an adsorptive dissociation of the prosthetic groups of certain enzymes from the apoenzymes.

Few investigations have been found of the general problem of the action of soaps upon enzymes. Velluz [1927] stated that fatty acids inhibit the action of pepsin, the effect increasing from C_6 to C_{18}; unsaturated fatty acids were more potent than the saturated ones, though with urease the latter were more active. Related to the question also seems to be the finding of Quastel [1932] that unsaturated glycerides increase the toxicity of the triphenylmethane series of dyes towards urease, though this action was rather specific.[1]

There is an evident relation also to the film penetration observations of Schulman & Rideal [1937] and Schulman [1937], though the nature of this relation is not yet defined.

Experimental

Ovoverdin. This was extracted from lobster eggs [Stern & Salomon, 1937] by grinding with sand and water and treatment of the fluid portion with an equal volume of saturated ammonium sulphate. After filtration, the ovoverdin was precipitated from the filtrate by more complete saturation with ammonium

[1] Upon completion of this work, there appeared a note in which Cook & Kreke [1938] state that fatty acids in a concentration of 0·5 mg./ml. stimulate the respiration of yeast, and at 1 mg./ml. stimulate skin respiration slightly. Higher concentrations were inhibitory. Such amounts cause massive depression in the pyruvate system here studied; a special further test showed that neither 0·1 mg. nor 0·05 mg. Na decoate had any stimulating effect with brain though these lie immediately below the concentration which has an inhibitory action, viz. 0·2 mg./3 ml.

sulphate, the precipitate collected upon a fluted filter paper, under cool conditions, and dissolved in 1% NaCl. Traces of ammonium sulphate were removed by dialysis against 1% NaCl. It was found to be important to keep the chromoprotein moist at all stages with dilute salt solution; drying or approximation to salt-free solutions rapidly causes the dissociative change from green to red. It is interesting in view of what follows that in its relation to salts and drying the chromoprotein follows closely in stability the pyruvate oxidase system in brain tissue, though there is a difference in sensitivity to freezing. This does not appear to affect ovoverdin, whereas it quickly destroys pyruvate oxidase.

Na salts of fatty acids. With the exception of the Na palmitate and oleate, which were of doubtful purity, the remaining soaps were obtained from commercial sources, but were subjected to several recrystallizations. The C_{10}, C_{12} and C_{14} acids were prepared from acids previously purified to the correct M.P. Some difficulty is experienced in obtaining solutions of the less soluble salts at the reaction required for test, approximately *p*H 7·4. It will be realized from the study of the *p*H relations at interfaces of the fatty acids [Peters, 1931; Wakelin, 1938] that at this reaction dodecoic acid tends to be present mainly as the acid and to leave an aqueous phase; decoate on the other hand shows a more marked water-solubility. Hence the use of a soap at this reaction must in reality mean the use of a mixture of salt and acid, and the really important point is to add it in a sufficiently soluble form to ensure that the mixture reaches the enzyme studied. In practice with dodecoate, tetradecoate and palmitate after solution in the appropriate amount of water, the whole was warmed, traces of phenol red added, and then HCl to give the desired *p*H. Where solution was possible only at 38°, the soap solution was kept in the warm bath until addition, together with a control containing water only. The soap and the water control were then added in similar volumes as a final addition to the Barcroft bottles. The highest concentrations used were Na octoate 0·1 *M*, Na decoate 0·045 *M*, Na dodecoate 0·047 *M* and Na tetradecoate 0·00206 *M*.

In other respects the respiration experiments with pigeon's brain tissue followed the usual technique of the laboratory; it will be recalled that the Ringer-phosphate medium (*p*H 7·3) does not contain Ca. The experimental volumes were 3·0 ml. in the Barcroft bottles; $1{\cdot}504 \times 10^{-3}$ *M* Na decoate = 1 mg. in 3·0 ml. In most experiments the figures quoted are the mean of duplicate estimations. The vitamin added was enough to produce maximum effects, 1·0–2·0 μg.; Na pyruvate, 6 mg. was added to each bottle.

Effect of Na salts upon ovoverdin

The minimum amount of Na salt necessary to cause dissociation was found by adding it in solution to 1·0 ml. of a suitable dilution of ovoverdin in 1% NaCl or in phosphate buffer (*p*H 6·7), until the green colour had disappeared. Table I shows the results with buffer present; in absence of the latter, the results were 20% higher but in the same sense.

Table I. *Amounts of Na salts of fatty acids required to cause dissociation of dilute ovoverdin solutions. Temp. 16–20°*

	$\times 10^{-3}$ *M*
Na octoate	>12·8
Na decoate	4·17
Na dodecoate	1·105
Na tetradecoate	1·042
Na palmitate	<1·2
Na oleate	1·09

The optimum concentration is reached with C_{12}; the difference between this and C_{14} is not significant. The figure for palmitate is unreliable owing to the low solubility. Since the change from water-solubility to lipoid-solubility takes place with a length of C chain of about 10, it seems to be clear that the effect is correlated with adsorption at the surface of an internal phase, and is explained if the acid displaces the astacin ester from its combination.

Reversibility. We may ask whether the acid interferes by combining irreversibly, or whether the effect is a loose displacement. It was not found possible to extract the fatty acid by any means which would leave the protein undenatured; but a simple addition of $CaCl_2$ to 0·02–0·04 *M* readily causes precipitation of such fatty acids under these conditions, and in the absence of phosphate will reverse the colour change to green. This is quite consistent with the idea that we are dealing with a reversible adsorptive phenomenon.

Effects upon some oxidation enzyme systems

The above observations are of interest in themselves, but they gain in importance by the finding that a similar action takes place upon a brain *brei*. The question has not been thoroughly explored; only with pyruvate and succinate as substrates has it been investigated, and with the latter not in detail. The interesting point is that with pyruvate there is inhibition increasing from the C_8 acid to the C_{12}, as with the dissociation of the lobster pigment. Fig. 1 illustrates the action of sodium decoate. In Table II the results for pyruvate are collected under the headings of the acid to which they refer. Where the actions of more than one acid were compared upon the same brain this may be seen from the no. of the exp. (column 6, Table II). In general the results for different exps. seemed to be sufficiently similar to make this the best method of examination. In the summary (Table II), the essential point is made that the inhibitory action increases from C_8 to C_{12}; after this the results for tetradecoate are of the same order, but are slightly complicated by solubility. Palmitate seems to be less active but the result is again rather uncertain, and the figure for concentration can only be regarded as a maximum one. With decoate and dodecoate amounts of 0·2–0·3 mg. produce definite effects. It is suggested in explanation that the acids displace an essential component of the oxidase system, as with the model ovoverdin. The experiments have been done throughout upon the avitaminous brain in order to obtain possible extra information; they are not complicated by the possibility that time was not allowed for combination of vitamin, because at least 5 min. were allowed after addition of vitamin and before adding the fatty acid. In many cases the inhibition for vitamin is greater than that for pyruvate alone; but this is probably not significant, because the figure for respiration without added vitamin has a relatively larger contribution from the residual respiration, which appears to be less inhibited.

In support of the idea that some coenzyme may be displaced from combination by the acid, we have found that with $4{\cdot}51 \times 10^{-3}$ *M* decoate the CO_2 evolution from pyruvate by washed yeast (acetone-treated) with added cocarboxylase is reduced by 35% both alone and in the presence of 10 μg. vitamin B_1; this experiment was carried out as described by Ochoa & Peters [1938].

The oxidase system for succinate in brain is more stable than that for pyruvate; the former survives freezing and extraction with distilled water. Similarly it is not much affected by fatty acid; a complete experiment upon this is given in exp. 12 with dodecoate; similar results have been obtained with decoate.

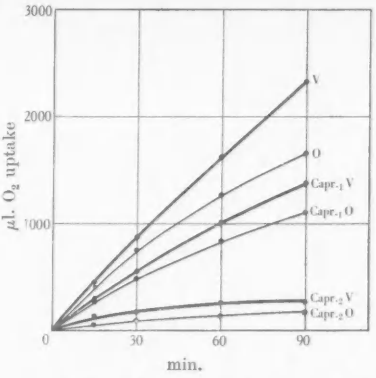

Fig. 1. The effect of small amounts of Na decoate upon the O_2 uptake of avitaminous pigeon brain in Ringer-phosphate pyruvate medium. Upper curves: V = with and O = without vitamin. Middle curve (capr.$_1$), addition of 0·2 mg. decoate. Lower curves (capr.$_2$), 0·7 mg. decoate.

Table II. *Inhibiting effect of Na salts of fatty acids upon rate of the O_2 uptake of pigeon's brain tissue (in presence and absence of vitamin B_1) with pyruvate, expressed as a decrease in μl./g./hr. and as a % decrease.*

	Conc. $M \times 10^{-3}$	No vitamin		Vitamin		Exp.
		μl.	%	μl.	%	
Octoate	16·0	−905	64·5	−1575	71·6	5
	4·8	−256	18·0	− 851	38·8	5
	1·9	+ 39	Nil	− 357	18·4	6
	1·6	+ 43	Nil	− 265	12·0	5
Decoate	5·27	−848	88·7	−1586	94·1	1
		−832	92·6	−1375	94·1	2
	4·51	−646	82·5	−1314	89·5	3
	1·5	−520	40·6	−1182	55·4	6
		−485	48·5	−1007	59·0	7
	1·05	−282	14·6	− 621	42·5	2
	0·31	− 3	Nil	− 210	14·4	2
		− 32	4·0	− 207	14·0	3
Dodecoate	4·94	−753	83·9	−1262	82·9	2
	1·65	−774	77·4	−1341	78·6	7
	0·99	−325	38·5	− 674	43·6	8
	0·424	−100	11·9	− 312	20·2	8
Tetradecoate	0·67	−211	31·2	− 516	33·6	10
	0·13	− 57	8·5	− 315	20·4	10
	0·33	− 28	3·5	− 175	13·3	11
	0·065	−115	15·0	− 160	12·1	11
Palmitate	0·6	− 79	10·6	− 226	15·8	9
	0·12	+ 18	Nil	− 102	7·2	9
Oleate	1·97	−578	60·5	−1217	72·2	1
	0·77	−523	54·7	− 917	54·4	1

The periods of respiration after start of the exp. upon which the above figures are based are as follows: 30–60 min., exps. 5, 7; 30–90 min., exps. 1, 2, 3, 6, 9; 30–120 min., exps. 8, 10, 11.

Summary

Acid	Conc. $M \times 10^{-3}$	Decrease % O	Decrease % V
C_{12}	1·65	77·4	78·6
C_{10}	1·5	48·5	59·0
C_8	1·6	Nil	12·0

Exp. 12. Avitaminous brain used so as to eliminate pyruvate oxidation to some extent in the residual oxidation. The figures quoted are for the period of respiration 15–90 min. NaD = Na dodecoate.

Substances present	Rate of resp. μl./g./hr.	Difference
No addition	968	
Succinate	2566	1598
$0{\cdot}705 \times 10^{-3}$ *M* NaD	470	
Same + Succ.	1922	1452
$1{\cdot}41 \times 10^{-3}$ *M* NaD	323	
Same + Succ.	1662	1339

Na succinate 0·082 *M*. Note that $1{\cdot}65 \times 10^{-3}$ *M* reduces respiration with pyruvate 77 % and 0·99, 38 %. The decrease in the residual oxidation was here 52 and 66 % respectively.

As would be expected from this, the succinic dehydrogenase from liver (pigeon) showed no reduction in activity upon adding Na dodecoate ($3{\cdot}4 \times 10^{-3}$ *M*). On the other hand exp. 13 shows that the system which causes dehydrogenation of pyruvate in brain is much affected.

Exp. 13. *Inhibition of dehydrogenase action by Na dodecoate.* The tissue from two normal pigeon brains was thoroughly mashed and extracted 6 times with 7 ml. 1 % KCl and 4 times with 7 ml. Ringer phosphate, *p*H 7·4. Care was taken to grind the whole thoroughly with a glass rod and to keep ice cold throughout but not frozen. Thunberg tubes were set up containing 50 mg. tissue, 0·2 ml. methylene blue (1:5000), 3 mg. Na pyruvate and 1·0 ml. Ringer-phosphate. The Na pyruvate was placed in the stopper and added after evacuation just before placing in the bath at 38°. One tube contained in addition 1 mg. Na dodecoate. Figures are quoted for one out of four similar experiments.

	Time for decoloration
Control	30 min.
Na pyruvate	9 min.
Na pyruvate with Na dodecoate	>150 min.

The results for the pyruvate dehydrogenase of pigeon's brain confirm those of Lipmann [1937]. It will be seen that the dodecoate has inhibited not only the pyruvate effect but also the residual reduction.

It might be thought that some of the phenomena described above were merely the result of the well-known cytolysing action of soaps, and consequent destruction of enzyme action intimately bound up with cell structure. This clearly could not apply to the ovoverdin, nor to the acetone-treated yeast; in the case of the pyruvate oxidase system, Dixon & Meyer [1936] have shown that many brain cells are destroyed in forming a *brei*; in confirmation and extension of this Dr Carleton has found that, in our brain *brei*, the cytoplasmic outlines of the cells are destroyed (unpublished). Hence the evidence is that here too the effect is on the enzyme system itself rather than the cell.

Discussion

There seems to be no doubt as to the practical importance of these observations. Where there is a possibility that fatty acids may pre-exist or be formed by lipase action or otherwise during the course of experimental procedure, it seems certain that they will influence adversely experimental results with isolated systems. In particular it is likely that they constitute some of the difference between the tissue *brei* and the slice. The insolubility at *p*H 5·5–6·0 of the inhibitor of brain glycolysis described by Geiger [1938] suggests an effect of this type. Again, this may be part of the reason why Quastel & Wheatley [1933],

and Jowett & Quastel [1935] found oxidation of fatty acids by slices of liver, but none with *brei*. If there is substance in the idea that the behaviour of ovoverdin serves as a model for the more loosely associated groups of enzymes, a new experimental tool is indicated. It has been pointed out previously [Peters, 1931] that the fact that fatty acids of the water-insoluble type ionize over the physiological range of *p*H is of biological significance. Here we find that part of the colloidal phase of a chromoprotein in solution is affected by the same type of acid. This shows that such interfaces exist in practice in such solutions. Further it is clear that the cell possesses yet another simple method of controlling its activities.

SUMMARY

Small amounts of the Na salts of saturated fatty acids (C_{10}, C_{12}, C_{14}) cause dissociation of the astacin ester from the protein compound in ovoverdin; this change can be reversed by Ca salts.

Similarly these soaps exert a markedly inhibitory action upon the pyruvate oxidase system; the effect upon the succinoxidase system is much less.

It is suggested that these phenomena are related.

We are indebted to the Medical Research Council and to the Rockefeller Foundation for financial assistance.

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CCXCIV. *S*-CYSTEINOSUCCINIC ACID

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(*Received 31 October 1938*)

We have reported previously [Morgan & Friedmann, 1938, 1] that thiol compounds react with maleic acid giving stable condensation products of the general formula $HOOC.CH_2.CH(COOH).SR$. Of the three substances investigated, thiolacetic acid, *l*-cysteine and glutathione, only thiolacetic acid gave an easily crystallizable reaction product. We have now succeeded in crystallizing the product obtained by the interaction of *l*-cysteine with maleic acid.

The new substance which we call S-*cysteinosuccinic acid* separates from methanol in hedgehog crystals, M.P. 134–135° after softening at 102° and from glacial acetic acid in needles, softening at 125° and melting at 134–135°. The analytical figures of both products were in agreement with the formula $C_7H_{11}O_6NS$, but the optical rotations were different. The substance from methanol was laevorotatory in aqueous solution whilst that from glacial acetic acid was optically inactive. It was found in fact that hot glacial acetic acid induced partial or complete racemization; thus the optical rotation of the laevorotatory substance from methanol ($[\alpha]_D = -29{\cdot}8°$), after one recrystallization from glacial acetic acid decreases considerably ($[\alpha]_D = -5{\cdot}1°$) and becomes zero after a second recrystallization from the same solvent. Simultaneously the form of the crystals changes from rosettes to grid needles.

The addition of *l*-cysteine to maleic acid involves the formation of a second asymmetric centre (**):

$$HOOC.CH_2.\overset{*}{C}\overset{**}{H}.(COOH).S.CH_2.\overset{*}{C}H(NH_2).COOH,$$

so that two optically active condensation products can result. So far we have no experimental evidence for the actual formation of a second optically active compound. The laevorotatory substance described in this paper is certainly the main, if not the only, reaction product. It can be isolated in a yield of 86·75% from the amorphous product resulting (93·5% yield) from the interaction of *l*-cysteine with maleic acid [Morgan & Friedmann, 1938, 1].

The directing influence of the first asymmetric carbon atom (*) on the configuration of the newly formed second asymmetric carbon atom (**) is in agreement with well known stereochemical observations. The actual configuration of this second asymmetric carbon atom (**) however must at present remain open.

The maleic acid reaction has been applied by us [Morgan & Friedmann, 1938, 2] to the study of enzyme reactions induced by SH-compounds. In the discussion of these results we pointed out that the conclusions based on the inhibition of an enzyme reaction by maleic acid must be checked by a direct method. We suggested Hopkins's SS, SH reaction [Hopkins, 1921] as suitable for this purpose [Hopkins & Morgan, 1938]. Recently v. Euler & Hellström [1938] have raised objections against the validity of conclusions based on Hopkins's reaction. We tried to supply the missing direct chemical evidence for an assumed interaction of maleic acid with SH-groups of enzymes or coenzymes. As SH occurs naturally, so far as we know at present, only as free

or combined cysteine, *S*-cysteinosuccinic acid should be formed by hydrolysis of enzymes or coenzymes after their reaction with maleic acid. This view has been shown to be correct for *S*-glutathionosuccinic acid. Hydrolysis of this compound gave *S*-cysteinosuccinic acid. The product was partially racemized.

Experimental

Crystallization of S-*cysteinosuccinic acid from methanol*

(1) 0·4 g. amorphous condensation product of *l*-cysteine and maleic acid [Morgan & Friedmann, 1938, 1] was dissolved in hot methanol. On cooling the solution slowly deposited white crystals. After removal of the supernatant fluid the crystals are washed with methanol and dried *in vacuo* at 45° over P_2O_5. (Found: C, 35·1; H, 4·6; N, 5·6; S, 13·1 %. $C_7H_{11}O_6NS$ requires C, 35·5; H, 4·6; N, 5·9; S, 13·5 %.) $[\alpha]_D^{21°} -29·75°$, $-29·84°$ ($c=2·236$ in water).

(2) 0·4 g. amorphous *S*-cysteinosuccinic acid was dissolved in approximately 30 ml. of hot methanol. Next day the solution was slowly taken down to 4 ml. under slightly reduced pressure. 0·347 g. of white hedgehog crystals separated which were washed with methanol. Yield 86·75 %. $[\alpha]_D^{21°} -27·58°$ ($c=2·357$ in water).

S-Cysteinosuccinic acid commences to shrink and soften at 102° and melts at 134–135° (decomp.). It is hygroscopic and the M.P. had to be taken after drying the substance in the melting tubes for several days *in vacuo* over P_2O_5. The substance is easily soluble in cold water, slightly soluble in cold, better in hot methanol. In hot glacial acetic acid it dissolves slowly and sparingly, apparently with decomposition which turns the solution yellow. It is not soluble in the other usual organic solvents.

Effect of glacial acetic acid on crystalline, laevorotatory S-*cysteinosuccinic acid*

(1) 0·1 g. crystalline *S*-cysteinosuccinic acid ($[\alpha]_D = -29·8°$) was dissolved in boiling glacial acetic acid. On cooling 0·0745 g. of crystals deposited quickly as a mixture of forms mostly needles with some rosettes $[\alpha]_D^{21°} -5·12°$ ($c=2·246$ in water).

Another sample prepared in the same way was analysed after drying *in vacuo* over P_2O_5 and KOH at 40°. (Found: C, 35·9; H, 4·8; N, 5·7; S, 12·8 %. $C_7H_{11}O_6NS$ requires C, 35·5; H, 4·6; N, 5·9; S 13·5 %.)

(2) 0·1 g. substance ($[\alpha]_D = -29·8°$) was dissolved in boiling glacial acetic acid and allowed to crystallize overnight. The crystals were redissolved in the mother liquor by boiling. On cooling and standing overnight 0·0430 g. needles of uniform appearance were obtained. The mother liquor was deep yellow and gave on concentration a gummy residue. M.P. of the needles was 134–135° (decomp.) after softening at 125°. $[\alpha]_D$ in water was zero.

The substance used for polarimetry was recovered and analysed after drying as above. (Found: C, 35·5; H, 5·0 %. $C_7H_{11}O_6NS$ requires C, 35·5; H, 4·6 %.)

S-*Cysteinosuccinic acid obtained by hydrolysis of* S-*glutathionosuccinic acid*

S-Glutathionosuccinic acid [Morgan & Friedmann, 1938, 1] was hydrolysed by Hopkins's [1929] method used for the hydrolysis of glutathione.

0·5 g. substance was boiled with 25 ml. of 25 % H_2SO_4 for 16 hr. The solution, diluted to 800 ml., was precipitated with $HgSO_4$ avoiding excess. The Hg precipitate separated slowly. After 2 days it was collected and decomposed with H_2S. H_2SO_4 was removed from the filtrate quantitatively with $Ba(OH)_2$, the solution transferred to a crystallizing dish and evaporated *in vacuo* over

H_2SO_4. Yield 0·21 g. Recrystallized from hot methanol, 0·090 g. hedgehog crystals were obtained. M.P. 134–135° after softening at 102°. For analysis the substance was dried *in vacuo* over P_2O_5 at 45°. (Found: C, 35·2; H, 4·8; N, 5·6 %. $C_7H_{11}O_6NS$ requires C, 35·5; H, 4·6; N, 5·9 %.) $[\alpha]_D^{21°} -6·43°$ ($c = 1·786$ in water).

The properties of the isolated substance were identical with those of *S*-cysteinosuccinic acid.

Summary

S-Cysteinosuccinic acid crystallizes from methanol in hedgehog crystals, softening at 102° and melting at 134–135° (decomp.).

On treatment with hot glacial acetic acid racemization occurs, apparently accompanied by partial decomposition.

S-Cysteinosuccinic acid is formed by hydrolysis of *S*-glutathionosuccinic acid.

The significance of *S*-cysteinosuccinic acid for the interpretation of the maleic acid reaction in enzyme work is pointed out.

We wish to express our thanks to Dr D. J. Bell for help in carrying out the micro-polarimetric experiments.

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CCXCV. INTERRELATIONS OF THE VITAMINS

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(*Received 24 October 1938*)

In recent years the possibility of an antagonism between vitamins A and C has often been mentioned. This antagonism, if it exists, might be revealed by the partial inhibition of the effects of vitamin C in scurvy by the simultaneous administration of vitamin A.

During the last few years we have had the opportunity of making a close study of vitamins A, B complex, C and D, and being somewhat sceptical about the existence of the above-mentioned antagonism we decided to make a thorough investigation of the subject. We wish to make it clear that when we speak of antagonism we have in mind harmful antagonism.

It is generally agreed that a balance should exist between the various vitamins. A state of equilibrium can be preserved in spite of considerable variations in the amounts of the vitamins supplied, but if one particular vitamin is present in too great an excess, the symptoms of hypervitaminosis will be the consequence. In such cases it may be possible to restore the health of the organism by supplying an adequate quantity of one or more of the other vitamins. The equilibrium between the vitamins is now re-established by the increased supply of the relatively deficient vitamins, an example of beneficial antagonism.

The existence of beneficial antagonism between the fat-soluble vitamins A and D on the one hand, and the water-soluble vitamins B and C on the other, appears to be unquestioned. Publications by workers such as Hopkins [1923], Harris & Moore [1928], von Euler [1933], Höjer [1926] and Light *et al.* [1929], reveal clearly its existence. We have therefore considered it superfluous to investigate this problem and have limited ourselves to a study of the existence of the possible harmful antagonistic action of vitamins A and D on vitamin C.

In studying the available literature in order to establish the grounds for the belief that there is a harmful antagonism between vitamins A and C, we have succeeded in finding only one publication which in our opinion seems to be reliable and which might be construed as revealing such an antagonism. This publication is by Mouriquand & Michel [1922], but even here there is no question of an actual antagonism between vitamins A and C, because these workers rightly limit themselves to the demonstration that cod liver oil under given conditions may have a destructive action on the antiscorbutic effect of vitamin C. We have therefore come to the conclusion that there is no evidence for such antagonism in the case of either man or the lower animals. On the other hand, there is evidence in favour of the existence of a synergistic action between these vitamins. Thus we might mention innumerable cases in which physicians prescribe cod liver oil and orange juice for infants and also the unquestionably good results obtained with various commercial preparations containing vitamins A and C.

Experimental

Our experiments were carried out with a liquid "Preparation X", composed chiefly of a concentrate of vitamins A and D, malt extract, orange juice, sugar and water. 1 ml. of this product (=1·32 g.) contained 800 I.U. vitamin A (i.e. 600 I.U. per g.), 120 I.U. vitamin D, between 5 and 5·5 I.U. vitamin C and also a little of the vitamin B complex. The ratio of vitamin A to vitamin C was therefore 800 to 5·3 or 150 to 1 in terms of International Units. Preparation X contained about 1 % of the above-mentioned vitamin concentrate.

Three series of preliminary experiments were made; in each series there were three groups of guinea-pigs, with four animals in each group.

The main object was to compare "Preparation X", which contained vitamins A and D as well as vitamin C, with "Preparation Y" which differed from Preparation X only in the fact that it contained no vitamin A or D. It was desired to ascertain whether vitamins A and D in Preparation X would inhibit the action of the vitamin C which it contained.

The daily diet in the three groups is shown in Table I.

Table I

Diet	Added vitamins	Ratio vitamin A : vitamin C
(1) Basal diet + 4 g. Prep. X	2400 I.U. vitamin A + 16 I.U. vitamin C	150 : 1
(2) Basal diet + 4 g. Prep. Y	23 I.U. vitamin A + 16 I.U. vitamin C	1·43 : 1
(3) Basal diet alone	No vitamin C	—

The basal diet was that of Tillmans and consisted of oats, milk, hay and a little cod liver oil, equivalent to a dose of 23 I.U. vitamin A and 3 I.U. vitamin D per diem. Preparations X and Y were given mixed in milk.

The animals in Groups 1 and 2 were fed on the basal diet alone until increase in weight ceased or loss of weight took place and were then given the preparations. Weights were recorded twice a week over a period of 50 days except in the case of Group 3 when the death of the animals occurred before the close of the testing period.

Following the preliminary experiments in which only a small number of animals was used in each group, work was carried out with a series composed of four groups with 10 animals in each group.

In order to compare the action of cod liver oil with that of Preparation X an extra group was included in this series. The animals in this extra group received the same quantities of vitamins A, D and C as those of the group receiving Preparation X, but vitamins A and D were given in the form of cod liver oil. Vitamin C was administered in the same way as in Group 2, namely in Preparation Y. Another reason for introducing this extra group fed on cod liver oil was to verify the experiments of Mouriquand & Michel [1922] in which they had used a basal diet with added orange juice and cod liver oil.

In order to have the same quantity of vitamin A (2400 I.U. vitamin A) in proportion to vitamin C as in the group fed on Preparation X, and so that the quantities of cod liver oil and of Preparation X should be of the same magnitude, we used 4 g. cod liver oil containing 600 I.U. vitamin A per g. The basal diet was the same as before. The cod liver oil was converted into an emulsion and mixed with the milk given to the animals.

The action of the vitamin C administered was assessed on the one hand by the increases in weight and the general condition and appearance of the animals, and on the other hand by post-mortem examinations of the animals which died or were killed and by X-ray photographs of the thorax.

We have prepared graphs showing the mean variations in weight for the groups of animals in each experiment, that is to say for the Preparation X group, Preparation Y group, and the negative control group in each of the three experiments of the preliminary series (see Figs. 1, 2 and 3) and for the Preparation X group, Preparation Y group, Y with cod liver oil group and the negative control group in the larger-scale experiment (see Fig. 4).

One animal fed on Preparation Y died from pneumonia. Three of the animals receiving cod liver oil died, probably from scurvy (post-mortem examination revealed scorbutic signs in each case). The negative control animals all died in less than 50 days with typical symptoms of scurvy.

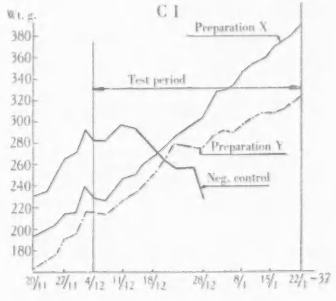

Fig. 1.

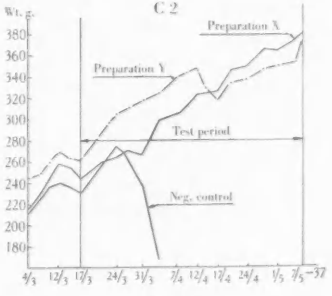

Fig. 2.

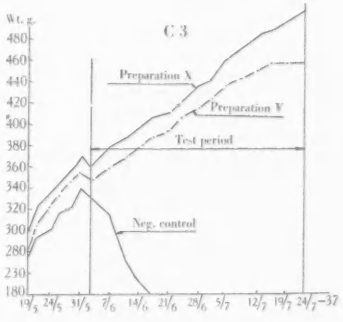

Fig. 3.

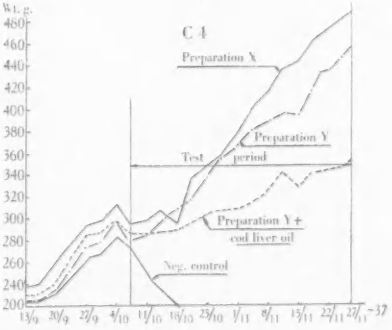

Fig. 4.

As can be seen from the curves, the mean increase in weight was in every series a little greater for animals receiving Preparation X (including vitamins A and D) than for those receiving Preparation Y (without added vitamins A and D). If, then, increase in weight is taken as a criterion of the effect of vitamins A and D on the action of vitamin C, the results of every experiment seem clearly to indicate that there is no antagonism between vitamins A and D and vitamin C. It will be noted that the weight increase of the animals receiving cod liver oil (Fig. 4), was less than that of those animals receiving Preparation X or Y.

In spite of the very large amount of vitamin A administered, large both from an absolute point of view and in proportion to the amount of vitamin C,

hypervitaminosis-A did not occur and the vitamin A appears to have reinforced the action of vitamin C.

The general appearance of the animals of the X groups was excellent, that of the Y groups very good, that of the cod liver oil group bad and the animals of the negative control groups were of course miserable in appearance.

Results of the autopsies

After the animals had died or had been killed at the end of the testing period, observations were made regarding the existence of haemorrhages and fragility of the bones and the condition of the thorax was examined. The results of these examinations are given below (Table II).

Table II. *Results of autopsies*

Series no.	Group no.	Preparation	Haemorrhages	Bone fragility	Scorbutic rosary
I	1	X	None	Normal	None
	2	Y	Almost none	Normal	Traces
	3	Negative control	Severe	Very fragile	Pronounced
II			Exactly as for Series I		
III			Exactly as for Series I		
IV	1	X	None	Normal	None
	2	Y	Almost none	Normal	Traces
	3	Y + cod liver oil	Rather pronounced	Rather fragile	Rather pronounced
	4	Negative control	Severe	Very fragile	Pronounced

All the animals in Group 1 were plump and healthy and the carcasses were light in colour.

The animals belonging to Group 2 (Preparation Y) were likewise in good condition, but they showed a few small haemorrhages, the colour of their carcasses was not so light as in Group 1, neither were they quite so plump. The bones were strong. X-ray examination of the thorax did not give quite so favourable a picture as in the case of Group 1. It could not be said that animals belonging to Group 2 exhibited distinct scorbutic symptoms and the difference between those receiving Preparation X and those receiving Preparation Y was very slight on the average. Any difference that existed was in favour of the "X" animals in Group 1.

All the negative control animals (Group 3 in the preliminary series I, II and III and Group 4 in the "Large Series" IV) showed typical symptoms of scurvy, severe haemorrhages, fragile bones (sometimes even breaking before death) and a pronounced scorbutic rosary.

Animals belonging to Group 3 in the "Large series" (cod liver oil) also showed symptoms resembling those of scurvy.

Mouriquand & Michel [1922] have shown that cod liver oil, under the conditions obtained in trials with guinea-pigs, can inhibit the action of ascorbic acid. However, we agree fully with the reservations made by them that one cannot conclude from these experiments on guinea-pigs that cod liver oil given in ordinary doses inhibits the antiscorbutic action of vitamin C in man. In this connexion it is interesting to note that Wieland [1936] denies the existence of any antagonism between vitamins A and C in human beings. It must be remembered that the doses of cod liver oil administered to the guinea-pigs in our experiments were extremely large. Taking into consideration the relative weights of the animals and man, the corresponding dose for a man would amount to nearly 1 kg. cod liver oil a day.

Stability of vitamin C in presence of vitamins A and D

It has been claimed that vitamin C may be destroyed by vitamin A *in vitro*. We have examined the chemical relations between vitamins A and D on the one hand and vitamin C on the other, using emulsions corresponding in their make-up to Preparations X and Y. The results of such investigations will not be given in detail on this occasion but they showed that vitamin C was not appreciably reduced in amount in either preparation 3 months after the date of their manufacture. After an interval of 6 months the diminution was 10–15%. These tests were made with a commercial preparation and no special precautions had been taken to prevent the destruction of vitamins. The analyses were made by Tillmann's method. Thus it was impossible to prove that vitamin C had been affected by the presence of vitamins A and D in the preparation.

SUMMARY

1. Using guinea-pigs as experimental animals it was found that vitamins A and D had no antagonistic action on vitamin C, even if the doses of the fat-soluble vitamins were very large in relation to the dose of vitamin C.
2. It seems possible that vitamins A and D can reinforce the action of vitamin C, even if the excess of vitamins A and D over vitamin C is very considerable.
3. Under the conditions of our *in vitro* experiments vitamins A and D had no destructive chemical action on vitamin C.
4. Very large doses of cod liver oil inhibited the antiscorbutic action of vitamin C in guinea-pigs.

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